PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
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in its capacity as elected Office

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Applicant CREEMERS, Jantina et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   11 February 2000 (11.02.00)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Nestor Santesso Telephone No.: (41-22) 338.83.38

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
159782		
International application No.	**International filing date** *(day/month/year)*	**(Earliest) Priority Date** *(day/month/year)*
PCT/NL 99/ 00453	15/07/1999	16/07/1998
Applicant		
CPRO-DLO et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

- [X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   - [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   - [X] contained in the international application in written form.
   - [ ] filed together with the international application in computer readable form.
   - [ ] furnished subsequently to this Authority in written form.
   - [X] furnished subsequently to this Authority in computer readble form.
   - [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).
3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 18

   - [ ] as suggested by the applicant.
   - [X] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

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Process to collect metabolites from modified nectar by insects.

Field of the invention

The present invention relates to isolated, purified DNA sequences which can act as promoters in eukaryotic cells. More specifically, the present invention is related to such DNA sequences which act as promoters to express genes in nectaries of plants. The present invention also relates to chimerical gene constructs comprising a structural or a synthetic gene under the control of a promoter that effects expression of said genes in nectaries. This invention also relates to a process for producing metabolites in honey by allowing insects, preferably bees, to collect and process nectar from plants that excrete said metabolites in nectar or other exudates. Further, this invention relates to plant cells, plants or derivatives therefrom, that express the said chimerical gene.

Background of the invention

Nectaries are nectar secreting organs or tissues that can be located inside (floral) or outside (extrafloral) the flower. The main component of nectar is sugar, the variation between nectars of flowers from different species mainly being the concentration and ratio of glucose, fructose and sucrose (Baker and Baker, 1982). In addition, depending on the plant species, varying amounts of polysaccharides, lipids, organic acids, volatiles, minerals, phosphates, alkaloids, amino acids and proteins have been detected

(Baker and Baker, 1982). Being a specialised sink organ, the nectaries are supplied with sucrose by phloem unloading (Davis et al., 1985, Hagitzer and Fahn, 1992).

The mechanisms of sugar accumulation and nectar secretion have been described for several plant species (Fahn et al., 1979). Sugar transport to the nectaries is achieved by active transport mechanisms and/or osmotic and chemical gradients. In the nectaries of many plants sucrose is converted to glucose and fructose, resulting in a hexose dominant nectar. Part of the hexoses are converted to starch, which is hydrolysed prior to anthesis and nectar secretion. Cell to cell transport of nectar in the nectary parenchyma tissue is mainly symplastic, as demonstrated by the presence of many plasmodesmata between these cells (Fahn et al., 1979). Nectar is secreted from secretory cells via the cell membrane (eccrine secretion) or via the Golgi and endoplasmatic reticulum vesicles (granulocrine secretion). Research on the molecular regulation of nectary development and nectary biochemistry has not been reported.

The main function of floral nectar is to reward pollinating insects. Insects collect nectar to meet their short-term energy requirements. Colony-living honeybees process large quantities of nectar into honey, which is stored in honey-combs of the beehive and is used as food supply during the winter period. Within the bee colony different classes of worker bees cooperate in the honey production process. Foraging bees collect pollen and nectar from the flowers and bring it to the hive. On returning to the hive they give most of it up to household bees. Pollen is used as a protein source, especially to feed the brood. Adult nurse and worker bees use little protein, their capacity to digest proteins being very low (Crailsheim et al., 1993). Honey processing takes place by repeated swallowing and bringing up of the nectar from the honey stomach. In the first process 15% of the water content is lost. This semi-

processed nectar is temporarily stored in a honeycomb cell and taken out later for further processing. The final process includes filtering the honey to discard small particles like pollen grains. Sugar metabolising enzymes (invertase, amylase) are added and the honey is concentrated to an average water content of 20%. Most nectars and honeys only contain traces of protein (<0.2%). However, *Calluna vulgaris* (heather) honey can contain up to 1.8% protein, giving it thixotropic properties (Butler, 1962). It is known that bees add enzymes like invertase to nectar during the honey processing. Therefore, the probability that proteases are also added is very low. Protein digestion does not take place in the honey stomach but in the intestine of the honeybee. However, the ability of adult worker bees to digest proteins is very low, their main requirement being energy which they obtain from nectar. Until now, it was not established which proteins are present in heather honey and whether these originate from floral heather nectar or are added to honey by honeybees.

In the present invention it was established that heather honey contains two unique proteins that originate from floral nectar of heather. Based on these results a production system for proteins in nectar and honey was established.

It is an object of the present invention to show that recombinant proteins can be secreted in nectar of transgenic plants, that this nectar is collected by honeybees and that the bees process this nectar into honey that contains the unaltered protein in a concentrated form.

Definitions

Honey: A substance that contains approximately 80% sugar and varying amounts of other components and that is produced by insects, preferably bees, that collect and process

nectar from floral or extrafloral nectaries, from honeydew, other plant exudates or artificial sugar solutions.

MADS box gene: a gene coding for a transcription factor having a region of 56 amino acids which is homologous to a similar region in the Arabidopsis AGAMOUS protein and Antirrhinum DEFICIENS protein. This region is called the 'MADS box'. At least 50% of the amino acids in this region should be identical to the amino acid composition in the MADS boxes of AGAMOUS and/or DEFICIENS.

Nectary: secretory organ or secretory tissue of plants, located in the flowers (floral nectaries) or outside the flower (extrafloral nectaries) that excrete nectar.

Nectar: sugar containing fluid that is secreted by nectaries. Nectar can also contain substances like minerals, amino acids, proteins, organic acids, volatiles, alkaloids etc.

Recombinant protein: the gene product of a recombinant DNA molecule.

Recombinant DNA molecule: A DNA molecule in which sequences which are not naturally contiguous have been placed next to each other by in vitro manipulations.

Promoter: The DNA region, usually upstream to the coding sequence of a gene, which binds RNA polymerase and directs the enzyme to the correct transcriptional start site.

Summary of the invention

The production of recombinant proteins for pharmaceutical purposes is a growing market. Until now, mainly bacterial and yeast systems have been used for bulk production of proteins. Recently animal production systems have also been

developed. With the availability of efficient transformation techniques for plants, procedures to use plants for the production of proteins are now in progress. In plants, the recombinant proteins are targeted to sink organs like tubers and seeds. A serious draw-back of these production methods is that the recombinant protein can only be obtained after extended, and therefore expensive, purification steps.

The present invention provides a method to produce metabolites, preferably recombinant proteins in honey, which is manufactured by insects, preferably honeybees, that collect floral nectar of transgenic plants. Harvesting of honey is very simple and purification of the protein is very straight forward and requires no advanced purification steps. To give an estimation of the protein yield in a crop like rapeseed, we suggest an average protein production of 2% in honey, as has been found in honey of heather. If one hectare of rapeseed yields 100-500 kilo honey in one season, a yield of 2 to 10 kilo protein can be obtained. In addition, the present invention provides a method to collect metabolites from honey that is derived from non-transgenic plants that secrete these metabolites in nectar. An example are secondary metabolites like acetylandromedol, a diterpine compound, that is excreted in nectar of *Rhododendron arboreum* and *Rhododendron barbatum* and of *Piptanthus nepalensis* (Martini et al., 1990).

This invention provides a gene from petunia, *NEC1*, that is highly expressed in the nectaries of petunia and weakly expressed in the stamens. It also provides another gene from petunia, *FBP15*, that encodes a MADS box protein and which is specifically expressed in the nectaries of petunia. Further, it provides the isolated DNA sequences of the promoters of the *NEC1* and the *FBP15* genes. Furthermore, this invention provides an isolated DNA sequence expressed in nectaries encoding a signal peptide that is translation-

ally fused to a germin-like protein (Lane et al., 1993, Dumas et al., 1995), having the function to target the mature germin-like protein to nectar of heather (*Calluna vulgaris*). This invention gives proof that protein-containing sugar solution is collected by honeybees to produce honey that has a higher protein content than the sugar solution itself, the protein having undergone no qualitative alterations. This invention also proofs that a recombinant protein can be produced in nectar of transgenic plants and that this protein is present in honey produced by honeybees that collected this nectar.

Accordingly, this invention provides an isolated DNA sequence which encodes a protein indicated NEC1 and having the amino acid sequence given in SEQ ID NO:1 of the sequence listing hereafter or homologs of NEC1. A homolog of NEC1 is predominantly expressed in nectaries and/or has at least 60% homology with the amino acid sequence given in SEQ ID NO:1. Further this invention provides an isolated DNA sequence which encodes a protein indicated FBP15 and having the amino acid sequence given in SEQ ID NO:2 of the sequence listing hereafter or a homolog of FBP15. A homolog of FBP15 is specifically expressed in nectaries and belonging to the MADS box family. Furthermore, a homolog is also a gene sequence that has at least 80% homology within the MADS box region and a 60% overall homology with the amino acid sequence given in SEQ ID NO:2. Further this invention provides the characterisation and the isolation of a DNA sequence which encodes a signal peptide indicated "CVSP" (Calluna vulgaris signal peptide), wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein that is expressed in nectaries, targeting of the protein to nectar.

The DNA sequences of the invention can also be characterised in that they comprise the *NEC1* gene and the *FBP15* gene having the nucleotide sequences given in SEQ ID NO:4 and

SEQ ID NO:5 respectively, or a functionally homologous gene or an essentially identical nucleotide sequence or part thereof or derivatives thereof which are derived from said sequences by insertion, deletion or substitution of one or more nucleotides, said derived nucleotide sequences being obtainable by hybridisation with the nucleotide sequences given in SEQ ID NO:4 and 5 respectively.

Furthermore, the DNA sequences of the invention can also be characterised in that they comprise signal sequence CVSP having the nucleotide sequence given in SEQ ID NO:6, or an essentially identical nucleotide sequence or part thereof or derivatives thereof which are derived from said sequences by insertion, deletion or substitution of one or more nucleotides.

Further, this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:1, or a homologous protein.
Furthermore, this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:2, or a homologous protein.

In a further aspect, the invention provides a protein encoded by any of the above defined DNA sequences. Further, the invention provides processes of producing transgenic plants exhibiting excretion of recombinant proteins in nectar, the expression of the chimerical genes and the targeting of the recombinant proteins being under the control of promoter sequences and a signal sequence as described in this invention. Still further, the invention provides processes of producing transgenic plants that produce recombinant proteins in nectar, the expression of

the chimerical genes being under the control of promoter regions upstream of other genes that are expressed in nectaries. Still further, the invention provides processes of producing transgenic plants that produce recombinant proteins in nectar, the expression of these proteins being under the control of any signal peptide that affects targeting of a protein in nectar.

Also, the invention provides recombinant double stranded DNA molecules comprising expression cassettes to be used in the above process. Further, the invention provides transgenic bacteria, transgenic plants producing recombinant proteins in nectar, and also plant cells, tissue culture, plant parts or progeny plants derived from said transgenic plants. Finally, the invention provides a process to produce recombinant gene products in honey, produced by bees that collect nectar from transgenic plants and process this nectar into honey.

Brief description of the figures:

Figure 1 shows a polyacrylamide gel with PCR products after Differential Display mRNA amplification. PCR reactions were performed with the oligo-dT primer T12MG in combination with 5 different random primers AP11-AP15 on cDNA samples of pistils without nectaries (two independent samples), nectaries (two independent samples), leaves and a mixture of sepals (s), petals (p) and stamens (a). Bold arrow depicts the cloned fragment DD18.

Figure 2 is the DNA sequence of the Differential Display RT-PCR clone DD18a. The primers prat 122 and prat 119 that were used for 5' RACE PCR reactions are underlined.

Figure 3 is the DNA sequence of clone RC8, obtained by RACE PCR with gene specific primers prat 122 and prat 119 (Fig. 2) in combination with adapter primers. Primer prat 129 (underlined) is used in the next step together with primer prat 122 to amplify the coding region of the *NEC1* cDNA.

Figure 4 is the full length sequence of *NEC1* cDNA. The translation start (ATG) and translation stop (TAA) are depicted bold.

Figure 5 shows the expression of *NEC1* (A) and *FBP15* (B) in wild type petunia plants (line W115) as determined by Northern blot analysis. Blot A contains total RNA, while blot B is enriched for mRNA. The tissues are indicated as: 1= leaf, 2= sepal, 3= petal, 4= stamen, 5= pistil, 6= nectary. For blot A the HindIII/EcoRI fragment of pDD18a was used as a probe. For blot B the full length cDNA of *FBP15* was used as a probe.

Figure 6 Expression of *NEC1* by in situ localisation of *NEC1* transcripts (A) and activity of the *NEC1* promoter in the nectaries (B) and the stamen (C) as shown by GUS expression driven by the *NEC1* promoter. The GUS assay used for the stamens was incubated overnight without modifications to prevent diffusion (example 8). The GUS assay for the nectaries was incubated for 5 hrs, using an assay mixture to prevent diffusion (example 8). For in situ localisation longitudinal sections of flowers of *Petunia hybrida* were hybridised with digoxigenin-labeled antisense *NEC1* RNA

Figure 7 is the DNA sequence from the promoter region upstream of a sequence encoding the NEC1 protein. Underlined is the translation start of *NEC1* cDNA.

Figure 8 depicts a schematic presentation of the T-DNA region between the borders of the binary vector pBNEP1, containing the *NEC1* promoter (Figure 7), the *GUS* reporter

gene and the nos terminator in pBINPLUS. This vector was used to generate transgenic plants to study the expression of the *NEC1* promoter.

Figure 9 shows the SDS-PAGE separation of proteins that are present in commercial honey samples from different flowers. M= marker, lane 1: wattle bark, lane 2: flower mixture, lane 3: heather, lane 4: clover, lane 5: rapeseed.

Figure 10 shows the SDS-PAGE separation of proteins that are present in commercial honey samples of rapeseed (RH2x, RH10x) and heather (HH2X, HH10X) and of nectar samples of rapeseed (RN2x, RN10x) and heather (HN2x, HN10x). M= molecular weight marker. Two (2x) or ten (10x) fold dilutions were used.

Figure 11 shows the SDS-PAGE separation of proteins present in dilutions of the sugar/BSA feeding solution (A) and of honey from bees that had collected the sugar/BSA solution (B) The dilutions of the sugar/BSA and honey/BSA solution was the same for both gels: 1= 15x, 2= 30x, 3= 60x, 4= 75x, 5= 75x, 6= 90x, 7= 105x, 8= 120x, 9= 135x. M= marker

Figure 12 shows the sequence homology of the N-terminal protein sequence of CVH29, a unique protein present in heather honey and nectar, with a germin-like protein GER1 from a gene bank homology search (BLAST).

Figure 13 shows the deduced DNA sequence of the N-terminal protein sequence of CVH29. The degenerated primers prat 176 and prat 177 are underlined (A). The DNA sequence of the PCR product obtained with prat 176 and prat 177 performed on genomic DNA of heather is shown in B. The gene-specific primers prat 207 and prat 206 used to perform 5'RACE PCR reactions on cDNA from heather flowers are underlined.

Figure 14 shows the DNA sequence of four independent clones obtained by 5'RACE PCR with prat 207 and prat 206 on cDNA of heather flowers. The ATG translation start of the putative signal sequence is boxed. The end of the putative signal sequence and the start of the mature protein are indicated by arrows.

Figure 15 is the sequence of the synthetically produced DNA molecule encoding the signal sequence CVSP (boxed) with linkers.

Figure 16 is the schematic representation of the plasmid pCV1. Not all restriction sites are indicated.

Figure 17 is the schematic representation of the plasmid pCV2. Not all restriction sites are indicated.

Figure 18 is the schematic representation of the plasmid pCV3. Not all restriction sites are indicated.

Figure 19 is the DNA sequence of the full length cDNA of *FBP15*. The translation start (ATG) and translation stop (TAA) are boxed. The MAD-box and K-box region are underlined.

Detailed description of the invention

This invention provides processes of producing transgenic plants that produce recombinant proteins in nectaries and nectar that is collected by foraging honeybees. This invention gives evidence that honeybees process protein containing nectar into honey that contains the unaltered protein in a concentrated form. Subsequently, the desired protein can be purified from the honey.

To express recombinant proteins in nectaries of transgenic plants, a translational fusion of an isolated DNA sequence from a promoter region upstream of a sequence encoding a protein that is expressed in nectaries with a sequence encoding the recombinant protein has to be carried out. Preferably, the isolated DNA sequence from a promoter region is upstream of a sequence that is specifically or highly expressed in nectaries.

The invention relates to a DNA sequence isolated from *Petunia hybrida* that encodes a protein indicated NEC1 or a homologous protein or part thereof. A homologous protein has at least 65% homology with the amino acid sequence given in SEQ ID NO:1. The cDNA sequence of the *NEC1* gene is given in Fig. 4 and in SEQ ID NO:4. The deduced amino acid sequence of the *NEC1* gene is given in SEQ ID NO:1. The *NEC1* gene shows strong expression in the nectaries and in a very localised region of the anther filaments of *Petunia hybrida*. The deduced amino acid sequence of *NEC1* predicts a membrane bound protein. The precise function of the gene has not been elucidated yet, but considering the phenotype of transgenic plants that ectopically express *NEC1* in the leaves, a role in sugar metabolism of *NEC1* is apparent.

The present invention also relates to homologous DNA sequences that can be isolated from other organisms, preferably plants, using standard methods and the already known DNA sequence of the *NEC1* gene. More precisely, it is also possible to use DNA sequences which have a high degree of homology to the DNA sequence of the *NEC1* gene, but which are not completely identical, in the process according to the invention. The use of sequences having homologies between 85 and 100 % is to be preferred. DNA sequences can also be used which result from the sequence shown in SEQ ID NO:4 by insertion, deletion or substitution of one or more nucleotides. This includes naturally occurring variations or variations introduced through targeted mutagenesis or

recombination. The DNA sequence shown in SEQ ID NO:4 can also be produced by using DNA synthesis techniques.

The invention also relates to a DNA sequence isolated from *Petunia hybrida* that encodes a MADS box protein indicated FBP15 or a homologous protein or part thereof. The cDNA sequence of *FBP15* is given in SEQ ID NO:5. *FBP15* shows exclusively expression in the nectaries of *Petunia hybrida*. The function of *FBP15* is unknown.
The present invention also relates to homologous DNA sequences that can be isolated from other organisms, preferably plants, using standard methods and the already known DNA sequence of *FBP15*. More precisely, it is also possible to use DNA sequences which have a high degree of homology to the DNA sequence of *FBP15*, but which are not completely identical, in the process according to the invention. The use of sequences having homologies between 85 and 100 % is to be preferred. DNA sequences can also be used which result from the sequence shown in SEQ ID NO:5 by insertion, deletion or substitution of one or more nucleotides. This includes naturally occurring variations or variations introduced through targeted mutagenesis or recombination. The DNA sequence shown in SEQ ID NO:5 can also be produced by using current DNA synthesis techniques.

Further, this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:1, or a homologous protein that is expressed in nectaries. Furthermore, this invention provides an isolated DNA sequence from the promoter region upstream of an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary specific sequence encodes a protein compri-

sing the amino acid sequence given in SEQ ID NO:2, or a homologous protein that is expressed in nectaries.

More specifically this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence has:
a) a nucleotide sequence given in SEQ ID NO:4, or
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

In a more specific embodiment this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:7, or a functional fragment thereof having promoter activity.

In a further aspect, the invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence has:
a) a nucleotide sequence given in SEQ ID NO:5, or
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

In a more specific embodiment this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:8 , or a functional fragment thereof having promoter activity.

Further, this invention provides an isolated DNA sequence comprising the coding region for a signal peptide, wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein

that is expressed in nectaries, targeting of the protein to nectar. More specifically, the DNA sequence comprises the nucleotide sequence given in SEQ ID NO:6 obtained from a plant of *Calluna vulgaris*, or a nucleotide sequence obtainable by hybridisation with the nucleotide sequence given in SEQ ID NO:6. The use of sequences having homologies between 95 and 100 % is to be preferred. DNA sequences can also be used which result from the sequence shown in SEQ ID NO:6 by insertion, deletion or substitution of one or more nucleotides. This includes naturally occurring variations or variations introduced through targeted mutagenesis or recombination. The DNA sequence shown in SEQ ID NO:6 can also be produced by using DNA synthesis techniques. The signal peptide CVSP was isolated from nectar of *Calluna vulgaris* flowers and from honey processed by honeybees that collected the nectar. The function of CVSP in heather nectaries is to target the germin-like protein to nectar. The DNA sequence CVSP can also be used to target other proteins to nectar in plant species.

A subject of the present invention is the use of DNA sequences for producing recombinant proteins in nectar of plants, wherein the protein is produced in nectaries and targeted to nectar, and wherein expression in nectaries is achieved by using a DNA sequence consisting of the promoter region upstream of a DNA sequence that is expressed in nectaries, and wherein secretion in nectar is achieved by using a DNA sequence that encodes a signal sequence that targets the recombinant protein to nectar. In a further aspect the present invention relates to processes wherein a recombinant protein is expressed in other plant tissues than the nectaries and wherein the biochemical composition of nectar is changed as a consequence of the recombinant gene expression. The present invention also relates to processes wherein a recombinant protein is expressed in nectaries of a transgenic plant, wherein the biochemical composition of nectar or the nectar secretion is changed as

a consequence of this protein expression. In particular, it relates to processes where the recombinant protein is an enzyme that interferes with the sugar metabolism in nectaries.

The production of a recombinant protein in nectaries and nectar is achieved by integrating into the genome of the plants a recombinant double-stranded DNA molecule comprising an expression cassette having the following constituents and expressing it:

i) a promoter functional in nectaries of plants,

ii) a DNA sequence encoding a protein which is fused to the promoter,

iii) a DNA sequence encoding a signal peptide that targets the recombinant protein to nectar, which is translationally fused to the DNA sequence encoding the recombinant protein, and optionally

iv) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

Such DNA molecules are also subject of the invention. The present invention provides an example of such a DNA molecule that contains the described expression cassettes in the form of plasmid pCV3 (Fig. 18), which comprises the promoter region of the *NEC1* gene from petunia, the signal sequence CVSP from heather, the coding region of the reporter gene *GUS* and the NOS terminator. In principle, any promoter that is active in the nectaries of plants can be used as promoter. The promoter is to ensure that the chosen gene is expressed in nectaries. Also, in principle, any signal sequence that targets the expressed protein to nectar can be used as a signal sequence. The signal sequence is to ensure that the protein is excreted in nectar. Furthermore, any sequence that encodes a recombinant protein in nectaries can be used in the present invention. Preferably, the subject of this invention relates to DNA

sequences that encode proteins to be used for pharmaceutical purposes. It is also possible to use the invention to produce proteins for other purposes, e.g. enzymes for biotests or antioxidants for food additives. Furthermore, it is possible to use the invention to produce metabolites in nectar that attract predators of pest insects or that kill or repel pest insects. In another aspect it is possible to use the invention to produce metabolites in nectar that modify the attractiveness of the plant for pollinating insects or improve the health of pollinating insects.

It is also possible to use DNA sequences that encode proteins that modify the nectar composition or the sink strength of nectaries. This means that the recombinant protein interferes with metabolic pathways in the nectaries, resulting in changed levels of compounds that are already present in nectar, or the formation of new compounds in nectar.

In addition, the present invention also relates to expression cassettes that contain the above mentioned DNA sequences, except for a signal sequence. The recombinant protein is then only expressed in the nectaries, but not targeted to the nectar Consequently, the expression of the recombinant protein in the nectaries can still affect nectar composition.

In a further aspect, the present invention also relates to expression cassettes that contain DNA sequences coding for a protein that is expressed in other tissues than the nectaries. The expression of the recombinant protein affects changes in the biochemical composition of nectar or in nectar secretion.

Finally, the present invention also relates to non-transgenic plants that produce metabolites in nectar that can be harvested and purified from honey that is produced by honeybees that collect this nectar. Examples for these metabolites are alkaloids, terpines, amino acids, proteins, pigments and volatiles.

A preferred embodiment of the process discussed above provides that the expression cassette is transformed to a plant species that produces nectar. Preferably, the recombinant protein is produced in nectar of plants that are visited by honeybees that collect the nectar. Honeybees collect floral as well as extrafloral nectar. The present invention relates to plants that produce recombinant proteins in floral or extrafloral nectar. In addition, the present invention also relates to plants that produce recombinant proteins in other plant organs, said plant organs producing an exudate that is collected by insects, preferably bees, and processed into honey. A particularly preferred embodiment of the present invention are plants that can be grown under controlled conditions. Controlled conditions are greenhouses or field facilities where transgenic plants can be grown according to the safety rules that are required. Preferably, the controlled conditions are such that bee colonies that perform normal foraging behaviour can be maintained in the same compartment during the flowering period. Preferred plants originate from the *Brassicaceae* family, in particular *Brassica napus*.

Examples

Example 1:
Cloning of *NEC1*

The *NEC1* cDNA was isolated using the mRNA Differential Display system (Genhunter Corporation, Brookline USA). The isolation of total RNA from nectaries, sepals, petals, stamens and pistils from open flowers and from young leaves of *Petunia hybrida* was done according to Verwoerd et al. (1989). Two independent RNA isolations were performed on nectaries as well as on pistils. A DNase treatment was carried out on each RNA sample, using the RNA MessageClean™ Kit (Genhunter Corporation Brookline USA, cat. No. M601). A

reverse transcription reaction was carried out on 0.1 µg RNA of each sample, using the oligo-dT primer T12MG from the Genhunter Kit. Following the protocol, PCR reactions were carried out using the arbritary primers AP11-AP15 in combination with primer T12MG from the Kit. The PCR products were loaded on a sequencing gel and after electrophoresis the gel was blotted on 3M paper, dried and exposed to X-ray film (Figure 1). Two adjacent nectary-specific bands were cut out from the blot and the DNA was purified according to the manual. Reamplification of the fragment was carried out using the oligo-dT primer T12MG and the arbritary primer AP15. After electrophoresis, the PCR product was extracted from the agarose gel by freezing the isolated fragment in liquid nitrogen, followed by centrifugation. DNA was precipitated by adding 1/10 volume 1% HAc, 0.1M $MgCl_2$ and 2.5 volume of 96% ethanol to the supernatant. The pellet was dissolved in 10 µl TE buffer. The fragment, now called DD10a, was cloned into a PMOSBlue T-Vector (RPN 1719, Amersham Little Chalfont UK) giving the vector pDD10a.

The nucleotide sequence of this 3' cDNA clone was determined by the dideoxynucleotide chain termination method (ABI PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, P/N 402078, Perkin Elmer) and is shown in Figure 2. The DNA fragment has a length of 460 nucleotides. The missing 5' part of the cDNA was isolated using the Marathon TM cDNA Amplification Kit of Clontech (catalog K1802-1) and following the procedure as described in the manual. Briefly, Poly A+ RNA was isolated from nectaries of *Petunia hybrida* flowers. After double stranded cDNA synthesis, adapters were ligated and a 5'RACE reaction was carried out using the adapter primer AP1 supplied in the kit and a gene-specific primer prat 122. The nucleotide sequence of prat 122 is: 5'-gtgggaaggctatgctacaagc-3' (Figure 2). The PCR product was diluted 10x and 1 µl was used in a second 5' RACE reaction with the nested adapter primer supplied by the kit (AP2) and the nested gene-specific primer prat 119

(Figure 2). The nucleotide sequence of prat 119 is: 5'-cctctccatggactgcaatgcg-'3 . After gel electrophoreses a fragment of ±850 bp was obtained that hybridised with clone DD18a. The fragment, now called RC8, was extracted from the gel, purified and cloned into a PMOSBlue T-vector as described above. The sequence is shown in Figure 3. The combined (overlapping) sequences of clones DD18a and RC8 are shown in Figure 4, comprising the full length cDNA of a gene called *NEC1* hereafter. The *NEC1* clone has a length of 1205 nucleotides and encodes for a polypeptide of 265 amino acid residues. Based on the deduced amino acid sequence, high homology was found with a cDNA that is associated with *Rhizobium*-induced nodule development in the legume *Medicago truncatula* (MtN3, gene bank number: gnl/PID/e274341). The percentages of identity and similarity are 47% and 72% respectively. Analysis of the predicted protein, using the CAOS/CAMM programme (Protein analysis 1991, Genetics Computer Group inc., Wisconsin USA), shows that the putative protein structure resembles membrane proteins, having six evenly spaced hydrophobic loops that traverse the cell membrane. In addition, a signal sequence is predicted at the N-terminus, while the C-terminus is highly hydrophilic. Highest homology with MtN3 is found in the N-terminal signal sequence, the first two membrane-spanning loops and the last two membrane-spanning loops. The C-terminal hydrophilic part shows the lowest homology (28% identity, 30% similarity). The function of *NEC1* has not yet been determined.

Example 2:
Cloning of *FBP15*

Petunia MADS box cDNA clones were isolated from a cDNA library made from nectaries of *Petunia hybrida* flowers. The cDNA library was constructed using the lambda ZAP cloning vector (Stratagene, La Jolla USA, catalog nr. 200400-

200402). The library was screened under low stringency hybridisation conditions with a mixed probe comprising the MADS box regions of Floral binding protein gene *FBP2, FBP6* and *pMADS3* (Angenent et al., 1993, 1994, Tsuchimoto 1993). The hybridizing phage plaques were purified using standard techniques. Using the in vivo excision method, E.coli clones which contain a double-stranded Bluescript SK- plasmid with the cDNA insertion between the EcoRI and XhoI cleavage site of he polylinker were generated. Cross-hybridisation of the purified clones revealed 3 independent clones that did not cross hybridise with previously isolated FBP cDNA's and which were designated *FBP15, FBP16* and *FBP17*. The nucleotide sequence of *FBP15* was determined by the dideoxynucleotide mediated chain termination method and is depicted in SEQ ID NO:5. The *FBP15* cDNA clone has a length of 1157 nucleotides and encodes a peptide of 222 amino acid residues. All characteristics of a MADS box protein are present in FBP15: a N-terminal located MADS box region which shows a high degree of similarity with other MADS box proteins, and a K-box in the middle of the protein with an alpha helical structure. *FBP15* is most similar to the tobacco MADS box protein NAG1, which is an Agamous homolog and expressed in whorl 3 and 4 (Huang et al., 1996, Mizukami et al., 1996).

Example 3:
Expression of *FBP15*

Expression of *FBP15* was determined by standard Northern blot hybridisation experiments. A DNA fragment comprising the complete cDNA of *FBP15* was used as a probe. High stringency hybridisation and washing conditions were used. Using 10 μg of total RNA from various petunia tissues, expression of *FBP15* was only detectable in nectaries. Using 10 μg of mRNA from various tissues, prepared by using the kit and protocol of the Quickprep Micro mRNA Purification

Kit (Pharmacia Biotech), expression of *FBP15* was only detectable in nectaries as shown in Figure 5B.
The expression in the ovary and nectaries was determined by in situ hybridisation using a DIG labelled antisense RNA probe corresponding to the full length cDNA of *FBP15*. In vitro antisense RNA transcripts were made using T7 RNA polymerase. A standard protocol for in situ hybridisation was used as described by Canãs et al., 1994. A hybridizing signal was observed evenly strong in all cells of the nectary tissue.

Example 4:
Expression of *NEC1*

The RNA expression of *NEC1* was determined by standard Northern blot hybridisation experiments. A DNA fragment comprising the complete sequence of the Differential Display clone DD18 (Figure 2) was used as a probe. Using 10 μg of total RNA from various petunia tissues, strong expression of *NEC1* was detectable in nectaries and weak expression in anthers. No expression was detectable in other floral organs, in leaves or in roots (Figure 5A).
The expression in the ovary and nectaries was determined by in situ hybridisation using a DIG labelled antisense RNA probe corresponding to the nucleotides 79 to 1036 of *NEC1* cDNA, comprising the coding region and part of the 3' untranslated region. A clone containing this sequence was obtained by PCR on adapter-ligated cDNA, using two gene-specific primers prat 122 and prat 129 (Figure 4). The nucleotide sequence of prat 122 is: 5'-gtgggaaggctatgctaca-agc-3'", comprising the nucleotides 1015 to 1036 of the *NEC1* cDNA. The nucleotide sequence of prat 129 is: 5'-gggatcc<u>atggcgcaattacgtgctgatg</u>-3', comprising the nucleotides 79 to 100 of the *NEC1* cDNA. The gene-specific region of the primers is underlined. The primer contains an extra

BamHI and NcoI site at the 5'end. A PCR fragment of 958 nucleotides was obtained and cloned into a PMOSBluc vector. The fragment was subcloned in a vector containing the T7 promoter and in vitro antisense RNA transcripts were made using T7 RNA polymerase. A standard protocol for in situ hybridisation was used as described by Canãs et al., 1994. Strong hybridizing signals were observed in the outer cell layers of the nectaries (Figure 6A)

Example 5:

Isolation of *NEC1* promoter fragment

The promoter fragment of *NEC1* was cloned using the genome walker protocol (PT3042-1) and kit as provided by Clontech Laboraties. Briefly, genomic DNA from *Petunia hybrida* was digested with 5 different blunt cutting restriction enzymes. GenomeWalker adapters were ligated and PCR reactions were carried out on each GenomeWalker "library" with a gene specific, reversed primer prat 148 and the adapter primer from the kit (AP1). The nucleotide sequence of prat 148 is: 5'-ccaagaaggccaaatatgaaagac-3' comprising the nucleotides 105 to 128 of the *NEC1* cDNA (Figure 4). PCR products were subjected to a second round of PCR, using the nested adapter primer AP2 and the nested gene specific, reversed primer prat 149. The nucleotide sequence of prat 149 is: 5'-aagtcatcagcacgtaattgcgcc-3', comprising the nucleotides 81 to 104 of the *NEC1* cDNA. From the second PCR a 2 kb fragment was isolated from the *StuI* library, which was cloned in the PMOSBlue T-vector, yielding the construct pMAS-10. Figure 7 (SEQ ID NO:7) shows the DNA sequence of the *NEC1* promoter in the construct pMAS-10, including the translation start of *NEC1* cDNA.

Example 6:
Construction of *NEC1* promoter-GUS

A PCR reaction was performed on pMA5-10 (example 5), using the forward vector primer U19 of pMOSBlue and the gene specific primer prat 169. The nucleotide sequence of prat 169 is:
5'-cgctgcagcgccatggtttttttagtgaagcccc-3'. The gene-specific region is underlined. The primer contains an NcoI and BglII restriction site at the 3' end. The PCR product was digested with KpnI and NcoI and ligated into a pBluescript-derived vector (pMO4) that contains the NTM19 promoter (Custers et al., 1997), the reporter gene *GUS* and the nos terminator. The KpnI/NcoI NTM19 promoter fragment was replaced, resulting in a *NEC1*-promoter/*GUS* translational fusion. The resulting plasmid pNEP1 was digested with SmaI to release the *NEC1* promoter/*GUS*/nos fragment and this fragment was ligated into a derivative of the binary plasmid pBIN (Bevan, 1984) yielding the binary plasmid pBNEP1 (Figure 8). pBNEP1 was introduced into *Agrobacterium tumefaciens* strain LBA4404 or C58pMP90 by electroporation. Plasmid DNA from the Agrobacterium transformants was isolated and the structure of the binary vector was verified by restriction analysis and PCR.

Example 7:
Generation of transgenic Petunia plants

Agrobacterium strain LBA4404 transformants were used to transform *Petunia hybrida* using leaf discs as described by Horsch et al. (1985). After shoot and root induction on kanamycin selection media, plants were transferred to soil in the greenhouse.

Example 8:
Histochemical GUS assay

Different plant parts of Kanamycin-resistant plants transformed with the pBNEP1 construct were analysed for the distribution of β-glucuronidase activity (GUS) using the method described by (Jefferson et al., 1987). In transgenic plants with high expression levels diffusion of reaction products to other tissues was observed. To avoid this spreading a modified GUS assay was used. Briefly, tissues were pre-treated with 90% cold acetone at -20°C for 1 h, then rinsed three times 20' with 100 mM phosphate buffer containing 1 mM potassium ferricyanide. After this treatment the standard GUS assay was performed with the modification that ferricyanide was excluded from the reaction mixture.

Example 9:
Results histochemical GUS assay

In very young flowers (<1,4 cm) no blue staining was observed, in flowers of 2-4 cm weak blue staining of the nectaries was observed. In flowers of (4-6 cm) strong blue staining was observed in the nectaries (figure 6B) and in a very restricted region of the upper part of the anther filaments (Figure 6C). GUS expression was highest in the outer cell layers of the nectary parenchyma. In cross sections of the anther filaments GUS expression was observed in all cells except in the xylem of the inner vascular bundle.

Example 10:
Protein analysis of heather honey and nectar

Samples of pure heather honey, together with samples of rapeseed, clover, wattle bark and lavender honey were diluted, dialysed and loaded on a 12% SDS page gel (Laemmli,

1970). All honey samples showed several identical high molecular weight protein bands. Heather honey contained 2 unique protein bands of 29 and 50 kDa (Figure 9). The proteins were named CVH29 and CVH50 (CVH stands for *Calluna vulgaris* honey). To determine the origin of the proteins, nectar and honey samples of rapeseed and heather were prepared and loaded on a 12% SDS page gel. The high molecular weight protein bands of around 70 kDa that are present in all honey samples were not observed in rapeseed or heather nectar (Figure 10). These proteins are added by honeybees during honey processing. Proteins CVH29 and CVH50 are present in heather honey and heather nectar, but not in nectar of rapeseed. Therefore, it was concluded that CVH29 and CVH50 are secreted in nectar of heather and can be recovered from honey derived from this nectar. The protein concentration in the heather honey we tested was around 0.5%.

Example 11:

N-terminal sequence analysis of CVH29 and CVH50

Honey samples were loaded on an SDS PAGE gel and after electrophoreses the gel was blotted on a PVDF membrane. After staining the CVH29 and CVH50 bands were cut out from the blot and N-terminal sequencing was performed on both proteins. The N-terminal sequence of CVH50 is: SVLDFCVADPS-LPDGPAGYSCTEPSTVTSQDF. The N-terminal sequence of CVH29 is: SVLDFCVADPSLPDGPAGYSCKEPAKVTVDDFVFHGLGTA. A gene bank homology search (BLAST) showed high amino acid sequence homology (63%) with germin-like proteins isolated from Arabidopsis (Figure 12).

Example 12:

Identification signal sequence of CVH29

Because the germin-like protein CVH29 is excreted in heather nectar it was expected that part of the cDNA encodes a signal sequence. Based on the N terminal amino acid sequence, degenerated primers were designed. The sequence of the forward primer prat 176 is: 5'-gayttyt-gygtngcngayce-3' (y= c or t, n= c, t, a or g). The sequence of the reversed primer prat 177 is: ccrtgraanacraartcrtc (r= g or a). A PCR reaction performed on genomic DNA of heather yielded a 99 bp DNA fragment. The fragment was sequenced and two reversed, gene-specific 5' primers were designed to clone the 5' cDNA by "Marathon cDNA racing" using the kit and protocol of Clontech laboratories (protocol PT1115-1, Clontech Palo Alto USA). The sequence of gene-specific primer prat 207 that was used is: 5'-ggtgactttagagggctccttgc-3', the sequence of gene-specific nested primer prat 206 is:
5'-gctccttgcaggagtagcctgc-3' (Figure 13). RNA was isolated from open flowers of heather and mRNA was prepared using the Pharmacia quickprep micro mRNA kit. After cDNA synthesis and adapter ligation a PCR reaction was performed, using the adapter primer AP1 and the gene-specific primer prat 207. The PCR product was used for a second PCR, using adapter primer AP2 and the nested gene-specific primer prat 206. A single fragment of around 300 nucleotides was obtained and cloned in a PMOSBlue T-vector. Four clones were sequenced. Figure 14 shows that three clones were identical and one clone had two different nucleotides in the untranslated 5' region. A putative signal sequence of 17 amino acids was identified between the ATG start codon and the first codon of the mature protein CVH29 that was identical in all four clones. The nucleotide sequence of the putative signal sequence (SEQ ID NO:6) is:
5'-atgtttcttccaattctcttcaccatttccctcctcttctcctcctcccatgct-3'.

Example 13:

Construction of an expression cassette for excretion of proteins in nectar

To clone the *NEC1* promoter into a PMOSBlue vector a PCR reaction was carried out on pMA5-10 (example 5) using the forward primer prat 247 and the reversed primer prat 248 (Fig. 7). Prat 247 contains an extra Pst1 restriction site. The *NdeI* restriction site of prat 248 coincides with the ATG translation start of *NEC1*. The nucleotide sequence of prat 247 is: 5'-ggctgcaggagtgttctttgatagaatg-3', the nucleotide sequence of prat 348 is: 5'-cgcca-tatgtttttttatggaagcccc-3'. Gene-specific regions are underlined. A 1,8 kb promoter fragment was obtained and cloned into a pMOSBlue vector, yielding the plasmid pNECP.

A DNA molecule encoding the signal sequence CVSP as depicted in SEQ ID NO:6 was produced by synthesis and subsequent annealing of two oligo molecules prat 245 and prat 246. The sequence of prat 245 is: 5'tatgttccttccaattcttttcactatttct-cttcttttctcttcttctcatgctctgttcttgatttc'3, the sequence of prat 246 is: 5'gatccgaaatcaagaacagaagcatgagaagaagagaaaagaa-gagaaatagtgaaaagaattggaaggaaca'3. The region encoding the signal sequence CVSP is underlined. To ensure correct cleavage of the signal peptide, the linkers were extended with the coding region for the first five amino acids of the mature germin like protein (Fig. 13). The codon usage of the signal peptide sequence was optimised for Arabidopsis. By addition of a BamHI restriction site at the 3' end, 2 extra amino acids were linked in frame to the mature protein. The resulting DNA molecule is shown in Figure 15. The fragment was ligated into a Nde1/BamH1 cut PMOSBlue vector, yielding the plasmid pCVSP.

pNECP was digested with NdeI and PstI to release the *NEC1* promoter fragment which was cloned into the Pst1/Nde1 cut

pCVSP, yielding the plasmid pCV1. A schematic representation of pCV1 is given in Figure 16.

A 250 bp long fragment containing the NOS terminator sequence (NOST) was obtained by PCR, using the forward primer prat 251 and the reversed primer prat 252 on DNA of pRAP 33, which is a pUC 19 derived plasmid. Prat 251 adds a SacI and XhoI site, prat 252 adds a SmaI and EcoRI site. The sequence of prat 251 is: 5'-gggagctcgagtcgttcaaacatttggcaataaag-3'. The sequence of prat 252 is: 5'-cgaattcccgggatctagtaacatagatgacac-3'. The NOST-specific regions are underlined. The PCR product was cloned into pCR-Script™ Amp SK(+) Cloning Kit (Catalog 21188-21190, Stratagene La Jolla USA), yielding the plasmid pCR-NOST. pCR-NOST was digested with Sac1 and EcoR1 and the resulting fragment was cloned into the pUC 19 (ClonTech), derived plasmid pUCAP yielding the plasmid pCVNOS.

The plasmid pGUSN358 was purchased from Clontech (catalog 6030-1) containing the reporter gene GUS in pUC 119, modified to destroy the N linked glycosylation site within the 1.814 Kb GUS coding sequence. A PCR reaction was carried out with gene-specific primers prat 249 and prat 250, yielding a fragment that contains the GUS gene coding region and a BamH1 restriction site at the 5'end and a Sac1 restriction site at the 3'end. The sequence of prat 249 is: 5'-ccggatccatgttacgtcctgtagaaacc-3'. The sequence of prat 250 is: 5'-gggagctcccaccgaggctgtag-3'. The GUS specific regions are underlined. Subsequently, the PCR fragment was digested with BamHI and SacI and ligated into the BamHI/SacI cut plasmid pCVNOS, yielding the plasmid pCV2. A schematic representation of pCV2 is given in Figure 17.

pCV1 is digested with PstI and BamHI and the resulting fragment is cloned into the PstI/BamHI cut plasmid pCV2, yielding the plasmid pCV3. A schematic representation of pCV3 is given in Figure 18. pCV3 is digested with AscI and

SmaI and the resulting fragment is cloned into a derivative of the binary plasmid pBIN, yielding the binary plasmid pBCV3. pBCV3 was transferred from *Escherichia coli* to the *Agrobacterium tumefaciens* strain LBA4404 and C58pMP90 by electroporation. The transformed Agrobacterium strain was used to transform Arabidopsis and petunia.

Example 14:
Protein production in nectar

Using the *GUS* reporter gene, GUS activity in nectar of transgenic plants was measured according to the method as described by Jefferson et al., (1987). Briefly, the assay was carried out by measuring the amount of methyl umbelliferone (MU) produced by GUS fluorometrically by emission of light of 455 nm. The absolute emission was corrected for artificial quenching using an internal standard of 1nM MU (Angenent et al., 1993).

Example 15:
Feeding experiments with honeybees

In September 1996 a beehive located outside was supplied with a 25% sucrose solution supplemented with 2% BSA (bovine serum albumin). After 3 weeks the bees had consumed 15 litters of the feeding solution and honey was harvested from the hive. Although the flowering season had mostly past, bees still foraged on flowers to collect nectar outside. Therefore, the honey produced during this period is derived from a mixture of the feeding solution and nectar from flowers. An SDS page protein gel was loaded with dialysed honey samples and sugar/BSA solutions. Figure 11 shows that the protein band of BSA was present in all the samples tested and no qualitative changes were observed in the honey samples compared to the sugar/BSA solutions. The BSA concentration in honey was 1.5 times higher than in the feeding samples, demonstrating that protein is concen-

trated in honey. Honeybees that foraged on the sugar/BSA solution did not show any aberrant behaviour and the colony developed normally.

Example 16:
Process of honey production from transgenic plants

Twohundred and fifty transgenic plants that each produce recombinant protein in nectar were grown in a greenhouse of 25 square meters. The facilities were adjusted according to the safety rules according to European law, including safety measures to prevent in- or outflow of insects. A beehive adjusted for small populations, containing around 200 worker honeybees and a queen, was placed in the greenhouse when the plants were flowering. When a queen is present, she will start laying eggs and larvae will come out. The presence of brood stimulates the bees to collect nectar and process it into honey. After 2-3 weeks bees processed the nectar into honey and stored in sealed cells of the honeycomb. Under the described conditions the amount of honey that can be harvested is 250-1000 grams.

Example 17:
Ablation of nectaries

By introducing the highly sensitive Rnase BARNASE in plant cells, under the control of a tissue-specific promoter, cell ablation can be achieved in very specific tissues or organs. Ablation of nectaries can be applied to decrease the attractiveness of plants for pest insects that forage on the nectar that is secreted by nectaries. In addition, plants without nectaries can be obtained that are more resistant to bacterial and fungal infections. An example is given for the ablation of nectary tissue by expressing bacterial BARNASE in nectaries, using the NEC1 promoter.

Plasmid DNA of pNEP1 (example 6) was digested with KpnI and NcoI to release the 1800 bp *NEC1* promoter fragment. The purified promoter fragment was ligated into a pWP90 derived vector, upstream of the BARNASE-BARSTAR bacterial operon construct (Hartley, 1988) The construct contains a 35SCaMV terminator of polyA signal cauliflower mosaic virus terminator sequence downstream of the BARNASE-BARSTAR operon. The resulting plasmid pWP126 was digested with KpnI/ XhoI to release the *NEC1*-promoter/BARNASE-BARSTAR/CamVpolyA fragment and this fragment was ligated into a pBIN-derived vector pBIN Plus. The recombinant vector was transferred via *Agrobacterium tumefaciens* (LBA4404) to petunia variety W115. Transgenic petunia plants were selected with flowers without nectaries or underdeveloped nectaries.

Many promoters are less specific than can be concluded based on promoter/GUS expression is concluded. Because the bacterial BARNASE is highly cytotoxic at very low concentrations it can be preferred to protect other plant tissues by expression of a ribonuclease inhibitor gene under the control of a weak, constitutive promoter (e.g. NOS promoter) or a tissue-specific promoter that is not active in the tissues where cell ablation is to be achieved (Mariani et al., 1992, Beals et al., 1997).

Example 18:
Ectopic nectary development

MADS box genes regulate floral meristem and floral organ identity. Ectopic expression of MADS box genes can change the developmental fate of floral organs or cells. Transgenic petunia plants ectopically expressing FBP11, an ovule-

specific MADS box gene, develop ovule-like structures on sepals and petals (Colombo et al., 1995). *FBP15* is a nectary-specific MADS box gene, involved in the molecular regulation of nectary development. In petunia nectaries develop at the base of the carpel. Ectopic expression of *FBP15* in petunia may result in the development of nectaries on other organs of the flower or on vegetative parts of the plant. An example is given of a gene construct that, when transformed to a plant, results in ectopic expression of *FBP15*.

FBP15 was amplified using a 5' primer that hybridises with *FBP15* sequences just upstream of the ATC translation start site and a 3' primer that hybridises with *FBP15* sequences just downstream the translation stop site. The 5'primer contains a NcoI recognition site, the 3'primer contains a BamHI recognition site. After the sequence was confirmed, the amplified *FBP15* fragment was inserted as a BamHI/NcoI fragment into the binary vector pCPO31. This binary vector was derived from pPCV708, as described by Florack et al. (1994), and contains three expression cassettes with a multiple cloning site between the left and right T-DNA borders. The cDNA was cloned in sense orientation between a modified CaMV 35S promoter and the nopaline synthase terminator sequence. The chimerical gene construct was transferred via Agrobacterium GV3101 to petunia variety W115, using the transformation method as described in example 7. Transgenic petunia plants were selected that show ectopic nectary development.

Example 19:
Modification of sugar composition and nectar secretion

Although sugar content of nectar from different petunia W115 flowers shows some variation, the ratio between hexoses and sucrose is very stable. Down-regulation or up-regulation of genes involved in the establishment of the

ratio between hexoses and sucrose in nectar will therefore modify nectar composition. An example is given for anti-sense expression of a petunia-derived invertase gene.

PCR primers were designed that hybridise with the cDNA of an invertase gene cloned from *Solanum tuberosum*. The 5' primer 5'-AAGGACTTTAGAGAGACCCCACCACTGCTGG-3' and the 3' primer 5'-AAATGTCTTTGATGCATAATATTTCCCATAATC-3' were used for a PCR reaction on genomic DNA of petunia to yield a fragment of around 420 bp. The fragment was sequenced and cloned into a pMOSBlue vector to used as a probe to screen a petunia nectary-specific cDNA library. Hybridizing phage plaques were purified and cDNAs were retrieved by in vivo excision as described in example 2. The expression of the cDNA's was determined by Northern blotting as described in example 3 and the sequence of a nectary-specific invertase was determined as described in example 2. The invertase gene was amplified using a 5' primer that hybridises with sequences just upstream of the ATG translation start site and a 3' primer that hybridises with sequences just down-stream of the translation stop site. Extra restriction enzyme recognition sites were generated to allow cloning of the cDNA in sense (overexpression) or antisense direction into the binary vector pCPO31 as described in example 18. The chimerical gene constructs are transferred via Agrobacterium GV3101 to petunia variety W115, using the transformation method as described in example 7. Transgenic petunia plants were selected that exhibit modified sugar composition in nectar.

Example 20
Modification of plant development

A DNA which is the *NEC1* gene or a homologous gene is introduced into a plant cell, the said DNA being induced by promoter elements controlling the expression of the introduced DNA in such a way that transcription produces

sense RNA. Plants were regenerated from the transgenic cells as described in example 7. Plants that ectopically express the *NEC1* gene exhibited modified leaf morphology and modified sugar composition. Furthermore, plants that ectopically express the *NEC1* gene showed a delay in flowering time.

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Sequences:

SEQ ID NO:1 amino acid sequence NEC1

1 MAQLRADDLS FIFGLLGNIV SFMVFLAPVP TFYKIYKRKS SEGYQAIPYM

51 VALFSAGLLL YYAYLRKNAY LIVSINGFGC AIELTYISLF LFYAPRKSKI

101 FTGWLMLLEL GALGMVMPIT YLLAEGSHRV MIVGWICAAI NVAVFAAPLS

151 IMRQVIKTKS VEFMPFTLSL FLTLCATMWF FYGFFKKDFY IAFPNILGFL

201 FGIVQMLLYF VYKDSKRIDD EKSDPVREAT KSKEGVEIII NTEDDNSDNA

251 LQSMEKDFSR LRTSK

SEQ ID No: 3 amino acid sequence of FBP15

Met Gly Arg Gly Lys Ile Glu Ile Lys Arg Ile Glu Asn Thr Thr Asn
1 5 10 15

Arg Gln Val Thr Phe Cys Lys Arg Arg Asn Gly Leu Leu Lys Lys Ala
20 25 30

Tyr Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Leu Ile Val Phe
35 40 45

Ser Ser Arg Gly Arg Leu Tyr Glu Tyr Ala Asn Asn Ser Val Lys Ala
50 55 60

Thr Ile Asp Arg Tyr Lys Lys Ala Ser Ser Asp Ser Ser Asn Thr Gly
65 70 75 80

Ser Thr Ser Glu Ala Asn Thr Glu Phe Tyr Gln Gln Glu Ala Ala Lys
85 90 95

Leu Arg Val Gln Ile Gly Asn Leu Gln Asn Ser Asn Arg Asn Met Leu
100 105 110

Gly Glu Ser Leu Ser Ser Leu Thr Ala Lys Asp Leu Lys Gly Leu Glu
115 120 125

Thr Lys Leu Glu Lys Gly Ile Ser Arg Ile Arg Ser Lys Lys Asn Glu
130 135 140

Leu Leu Phe Ala Glu Ile Glu Tyr Met Arg Lys Arg Glu Ile Asp Leu
145 150 155 160

His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
165 170 175

Asn Val Asn Met Met Gly Gly Glu Phe Glu Leu Met Gln Ser His Pro
180 185 190

Tyr Asp Pro Arg Asp Phe Phe Gln Val Asn Gly Leu Gln His Asn His
195 200 205

Gln Tyr Pro Arg Gln Asp Asn Met Ala Leu Gln Leu Val
210 215 220

SEQ ID NO:3 amino acid sequence CVSP

MFLPILFTISLLFSSSHA

SEQ ID NO:4 Nucleotide sequence *NEC1*

```
1     TCGAGCGGCC GCCCGGGCAG GTATTCAACA AGAGTATTCA CCACTTGAAC
51    TCAAAAGGGG CTTCACTAAA AAAAAATCAT GGCGCAATTA CGTGCTGATG
101   ACTTGTCTTT CATATTTGGC CTTCTTGGTA ATATTGTATC ATTCATGGTC
151   TTCCTACCAC CCGTGCCAAC ATTTTACAAA ATATATAAAA GGAAATCATC
201   AGAAGGATAT CAAGCAATAC CATATATGGT AGCACTGTTC AGCGCCGGAC
251   TATTGCTATA TTATGCTTAT CTCAGGAAGA ATGCCTATCT TATCGTCAGC
301   ATTAATGGCT TTGGATGTGC CATTGAATTA ACATATATCT CTCTGTTTCT
351   CTTTTACCCC CCCAGAAAGT CTAAGATTTT CACAGGGTGG CTGATGCTCT
401   TAGAATTGGG AGCCCTAGGA ATGGTGATGC CAATTACTTA TTTATTAGCA
451   GAAGGCTCAC ATAGAGTGAT GATAGTGGGA TGGATTTGTG CAGCTATCAA
501   TGTTGCTGTC TTTGCTGCTC CTTTAAGCAT CATGAGGCAA GTAATAAAAA
551   CAAACAGTCT ACACTTCATC CCCTTCACTT TATCTTTGTT CCTCACTCTC
601   TGTGCCACTA TGTGGTTTTT CTATGGGTTT TTCAAGAAGG ACTTTTACAT
651   TGCGTTTCCA AATATACTGG GCTTTCTATT CGGAATCGTT CAAATGCTAT
701   TATATTTTGT TTACAAGGAT TCAAAGAGAA TAGATGATGA AAAATCTGAT
751   CCTCTTCCAC AAGCTACAAA ATCAAAACAA GGTGTAGAAA TCATTATCAA
801   CATTGAAGAT GATAATTCTG ATAACGCATT GCAGTCCATG GAGAAGGATT
851   TTTCCAGACT GCGGACATCA AAATAAGCAA GAAGATGATC AAAAAATGAC
901   AAAGCTAAGG AGTTTGAAGT AAGGCAAGGA ACTTGACACT GAATATCTAA
951   GCTAATTAGC AAGACTTTAG CAGCTTGTAA TATTTAGTGT TTGTGACGTG
1001  TTACCTTATA ATTAGCTTGT AGCATAGCCT TCCCACTAAT AATTCTGCTT
1051  AGCGAATCTT ATATATGGGA AATACTTACA CTAGTATGCA TCTTCTATAT
1101  ACATGTTTGG CACTTGACTA TACATAGAAA AATTAACAAG CATTTCTCAC
1151  CTCAATTTGT CACTTACTTA TAAGTAGCTG AATAATATAA TGCAATTTTC
1201  ACCCC
```

SEQ ID NO: 5 Nucleotide sequence *FBP15*

```
1     TCTGAATACAAGCTGTGTGTGTAGAGAGATTTCATAAAGACAGCAAACAT
51    CCCTTCTTTTTGTTCTGTTTTAAAAGTTCCCTTCTTCAACCAGCTCTTTT
101   CCTCATCAGGGTAAGTTGCAAATAAACCGGATGTTCCAGAATCAACAAGA
151   GAAGATGTCAGACTCGCCTCAGAGGAAGATGGGAAGAGGAAAGATTGAGA
201   TTAAGAGGATTGAAAATACAACAAATCGTCAAGTCACTTTCTGTAAGAGA
251   ACAAATCCCTTGCTTAAAAAAGCTTATGAACTTTCTGTTCTTTGTGATGC
301   TGAAGTTGCTCTCATCGTTTTCTCAACCCCTGGCCGCCTCTATGAATATG
351   CTAACAACAGTGTGAAGGCAACAATTGATAGATATAAGAAAGCATCCTCA
401   GATTCCTCCAACACTGGATCTACTTCTGAAGCTAACACTCAGTTTTATCA
451   ACAAGAAGCTGCCAAACTCCGAGTTCAGATTGGTAACTTACAGAACTCAA
501   ACAGGAACATGCTAGGCGAGTCTCTAACTTCTCTGACTGCAAAAGATCTG
551   AAAGGCCTGGAGACCAAACTTGAGAAAGGAATTAGTAGAATTAGGTCCAA
601   AAAGAATGAACTCCTGTTTGCTGAGATTGAGTATATGCGAAAAAGGGAAA
651   TTGATTTGCACAACAACAATCAGATGCTTCGGGCAAAGATAGCTGAGAGT
701   CAAACAAATGTGAACATGATGGAGGACAATTTGAGCTGATGCAATCTCA
751   TCCGTACGATCCAAGAGACTTCTTCCAAGTGAACGGCTTACACCATAATC
801   ATCAATATCCACGCCAAGACAACATGGCTCTTCAATTAGTATAAGTTTAT
851   AATAAAATGCATGGTTTGAAGCACTCTGATTGTGGTGGATTTGGATTATG
901   TATAACGGAGTGCAGGCCATTTGCCAATTATTGAAAGGTACTCAAACAGG
951   AAGTTGAAGAAGTTCATCATCTCTCTCATCTATATGTCTTAACAAAAGTC
1001  TTAGCTTATGGACTCTAAAACAAAGACTTAATTTAACATATAAATATAAT
1051  TGTGTAATGCTGTTGTATTGTATGGTATGTATCCAAAAACATTAATAACC
1101  TATCTTTTTCTTCAAATTATGTCTCCTTTGATACAAACTACTAACATATT
1151  TTCTTAT
```

SEQ ID NO:6 Nucleotide sequence CVSP

ATGTTTCTTCCAATTCTCTTCACCATTTCCCTCCTCTTCTCCTCCTCCCATGCT

SEQ ID NO:7 Nucleotide sequence *NEC1* promoter

1	CCTAGGAGAAATCAAGCCTACTCTTAAGATGGATGACTCACTTGCCCCGA
51	TGGTAAGGTGAAGGATCTGTTGATTAGAGTTGGGAAGTTCATGTTCTCTG
101	CTGATTTTATTATTCTAGACTATGAAGAGGACCAAGAAGCTCCAATAATT
151	TTGGGAAGAGCATTCTTAATCACATCGATGGCAATTATTGACATGGAACT
201	TGGGGAGATGACTGTGAGAGCGCATGGAGAAAAGGTTACTTTCAACCTTT
251	ATAATAAAAAGGATCATATGCCTAACTTTGAAGAGTGTTCTTTGATAGAA
301	TGTGTCAGACCACAACATCAAAGTAAACCGAAAGAGGTGTTTGAGCGGAA
351	TCTACAACAAAGTGACCACGGCACAATAATTGACAAGTTGAAGGAAAATT
401	CACCTAAAGGAAGGAAGAAGACAAAAGTTCGTCGTAACAAGAGGAGACGT
451	AAATGCTGGAAGTGAGCTTAAAGGTGTTGTCGTACTACGACGTTAACTAA
501	GGCGCTTGTCGGGAGGCAACCCTAGCTTTGTATGTAAATGTAAAAGTAAA
551	AAATATATATATAGAAAAAGGAAAATACAAAAAGAGTCGTGCCGCGACGT
601	TAAATCAAGCGCTTGTTGGAAGGCAACCCAATTTTTATTGTTTTAGTTGT
651	TTTACTTATTTAGTATTACGTAGTTTCTTGTTGTTTTTGTAGGGCTCGGG
701	ACTTTCGGAAGGTGAGGTAATTTCAAGGCATCGCGGTGTGTATTGCACCC
751	AGGTAAGTGTAAGAGTTGAGTTGGAAGCGTTTGGCCAAGTCTTGCACCGT
801	GAGAGGCTTTCAACCTGTTCCCACACGTGAAAAATTAAGAGCCAGATCTG
851	CTACATTAGCACTGAAGCATCGCTTGGCCAATAGCTTGGAATGGAAGCAA
901	GAATTCAAACCAAAATCAGAAACGCCACAAGAGATGTGTCGCACACTGCA
951	AAGCTTTGTGCAAACTAGTGAACGCAGAAATAGAAATGCTACAGCCCATG
1001	CGTCGCTTGGCTTATGGCAGGCAGCAAAAATTCAGCAGCAAAACAGAAAC
1051	GCTGCGAGAAACGCGTCGCATACGCCATAGCTTTGTGTCAAACAGAACGT
1101	CCAGAAATTGAAAAGCTATAAGCCTGCGTCGCTTGGCTCATGGCGTGCAG
1151	ACTAGAAAAGCTCTAGCAGATGCGTCGCGTATTGTATAGCTTGGTGTGAA
1201	ACAGAAAGTTCGAAACTTGGAAAACGATAACCCAGCGTCCCCTCTTCAAC
1251	CGCGTCCAGGTAAGTTCAAGATTCTTACGGGTTGACCCATTAACCCATTG
1301	ATCGGCTCATTATAAACAATAAAACATCACCTTCAACTATCACATGATTT
1351	CATAACTTTGACCTAGGATATTTTATATATATATATATATATATACACAC
1401	ACACACCATTTCCACCCATCTTACCTCATTTTTATTCAAACCATTTTTCT

1451 GCTTCAAAAGTTTAAATTATTAATATGATAAGTCATCCATAGTCAAACAA

1501 GATTTTCTATACTATTTTGTCCCTTGTAATTTTAAAAAAAAAATGAGCGA

1551 TGGTAAGATAAACATTGTTTGCAAGTGTACAATTTTAGTATATGCAAACC

1601 AACGCTTCTTCTTCCAACTATCACCTAAAACTACATCATTTATGGCGGGC

1651 GGACTACACGTAGCCAAATATAAAAACGCAATGGCCATTCAGTTCATGTC

1701 ATTTTTATATCCTTCATCCAATAATATTACTCAAAATTGATGTACAGTTT

1751 GCTCTCTGATGTGCACTTTACTATACGTAATACGGAATTTACATTATAAT

1801 TAAAGAGAACTGTTCCACTAAATTTTAATGATTTAATTAATTTAACTCGG

1851 TTACTTGTATTATTATTATTGCTGTATTTGTTTGTCATTTGAATTTGGCA

1901 CCGCAGATTTTTGTATCCAATTAACCCTCATATATCTTTTGGCCAAATAA

1951 AGAAAAAGTCTGCATATTTCTTGCCAAACATTTATCATACTTTACCGAAT

2001 TCTTGTTTTTTGTTTCTCTGTTGTTGTTCTCCACTATAAATAACATTTGC

2051 AGTGAGTAAAGTTTCTTCAGGTCTCTTTTGTAGATTCAACAAGAGTATTC

2101 AGCACTTGAACTCAAAAGGGGCTTCACTAAAAAAAATCATG

SEQ ID NO: 8 Nucleotide sequence *FBP15* promoter

Claims

1. An isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO: 1, or a homologous protein.

2. An isolated DNA sequence according to claim 1, wherein the nectary-specific expressed sequence has:
a) a nucleotide sequence given in SEQ ID NO:4, or
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

3. An isolated DNA sequence according to claim 1 or 2, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:7, or a functional fragment thereof having promoter activity.

4. An isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:2, or a homologous protein.

5. An isolated DNA sequence according to claim 4, wherein the nectary-specific expressed sequence has:
a) a nucleotide sequence given in SEQ ID NO:5, or
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

6. An isolated DNA sequence according to claim 4 or 5, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:8 or a functional fragment thereof having promoter activity.

7. An isolated DNA sequence encoding a protein comprising the amino acid sequence given in SEQ ID NO:1, or a homologous protein, the expression of the DNA sequence being predominantly confined to the nectaries of a plant.

8. An isolated DNA sequence according to claim 7 having:
a) a nucleotide sequence given in SEQ ID NO:4, or
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

9. An isolated DNA sequence that results from the sequence shown in SEQ ID NO:4 by insertion, deletion or substitution of one or more nucleotides, including naturally occurring variations or variations introduced by targeted mutagenesis or recombination.

10. An isolated DNA sequence according to claim 7 having a nucleotide sequence given in SEQ ID NO:4, said sequence being produced by current DNA synthesis techniques.

11. An isolated DNA sequence encoding a protein comprising the amino acid sequence given in SEQ ID NO:2, or a homologous protein, the expression of the DNA sequence being predominantly confined to the nectaries of a plant.

12. An isolated DNA sequence according to claim 11, having:
a) a nucleotide sequence given in SEQ ID NO:5, or
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

13. An isolated DNA sequence that results from the sequence shown in SEQ ID NO:5 by insertion, deletion or substitution of one or more nucleotides, including naturally occurring variations or variations introduced by targeted mutagenesis or recombination.

14. An isolated DNA sequence according to claim 11 having a nucleotide sequence given in SEQ ID NO:5, said sequence being produced by current DNA synthesis techniques.

15. Any sequence that encodes a nectary-specific MADS box gene.

16. An isolated DNA sequence comprising the coding region for a signal peptide, wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein that is expressed in nectaries, targeting of the protein to nectar.

17. An isolated DNA sequence according to claim 16, comprising the nucleotide sequence given in SEQ ID NO: 6 obtained from a plant of *Calluna vulgaris*, or a nucleotide sequence obtainable by hybridisation with the nucleotide sequence given in SEQ ID NO:6.

18. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in plants,

ii) a DNA sequence coding for a protein as defined in any of claims 7 to 15 which is fused to the promoter sequence in sense or antisense orientation, and optionally

iii) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

19. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in nectaries of plants,

ii) a DNA sequence coding for a protein which is fused to the promoter sequence in sense or antisense orientation, and optionally

iii) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

20. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in nectaries of plants,

ii) a DNA sequence encoding a protein which is fused to the promoter,

iii) a DNA sequence encoding a signal peptide that targets the recombinant protein to nectar, which is translationally fused to the DNA sequence encoding the recombinant protein, and optionally

iv) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

21. A recombinant double-stranded DNA molecule according to claim 19 or 20 wherein the promoter is as defined in any of claims 1-6.

22. A recombinant double-stranded DNA molecule according to claim 20 or 21 wherein the DNA sequence encoding a signal peptide is as defined in claim 16 or 17.

23. A process for producing a transgenic plant exhibiting excretion of a recombinant protein in its nectar, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 20 to 22, wherein the recombinant protein is excreted in nectar

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

24. A process for producing a transgenic plant exhibiting a modified nectar composition, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 19 to 22,

wherein the recombinant protein interferes with metabolic pathways in the nectaries,

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

25. A process for producing a transgenic plant exhibiting a modified nectar secretion, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 19 to 22, wherein the recombinant protein interferes with sink strength of nectaries

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

26. A process for producing a transgenic plant exhibiting a modified nectary development, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in claims 19 or 22, wherein the recombinant protein interferes with the development of nectaries

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

27. A process for producing honey from modified nectar of transgenic plants, comprising:

i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule as defined in any of claims 19 to 22, regenerating plants from the transgenic cell, and selecting modified plants exhibiting the excretion of nectar with a modified composition,

ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey.

28. A process for producing a recombinant gene product from honey, comprising:

i) producing a transgenic plant by introducing in a plant cell a recombinant double stranded DNA molecule as defined in any of claims 20 to 22, regenerating plants from the transgenic cell, and selecting modified plants exhibiting excretion of the recombinant gene product in nectar,

ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey, and

iii) isolating and purifying the gene product from the honey.

29. A process for producing a metabolite from honey, comprising:

i) producing a plant that excretes this metabolite in nectar and which plant has been produced by current breeding and selection methods,

ii) allowing insects, preferably bees, to collect nectar from the selected plants and to process the nectar into honey, and

iii) isolating and purifying the metabolite from the honey.

30. Micro organisms containing DNA sequences according to one or more of claims 1 to 17.

31. Micro organisms containing recombinant DNA molecules according to any of claims 18 to 22.

32. A plant cell or plant cell culture transformed with one or more DNA sequences according to claims 1 to 17.

33. A plant cell or plant cell culture transformed with recombinant DNA molecules according to any of 18 to 22.

34. A plant consisting essentially of the plant cells of claims 32 or 33.

35. A transgenic plant obtained by the process of any of claims 23 to 26.

36. Seeds, tissue culture, plant parts or progeny plants derived from a transgenic plant according to claim 35.

37. Honey obtained from nectar from transgenic plants, which nectar has a modified composition.

38. Honey obtained from nectar from transgenic plants, which nectar comprises a recombinant gene product.

09/743885

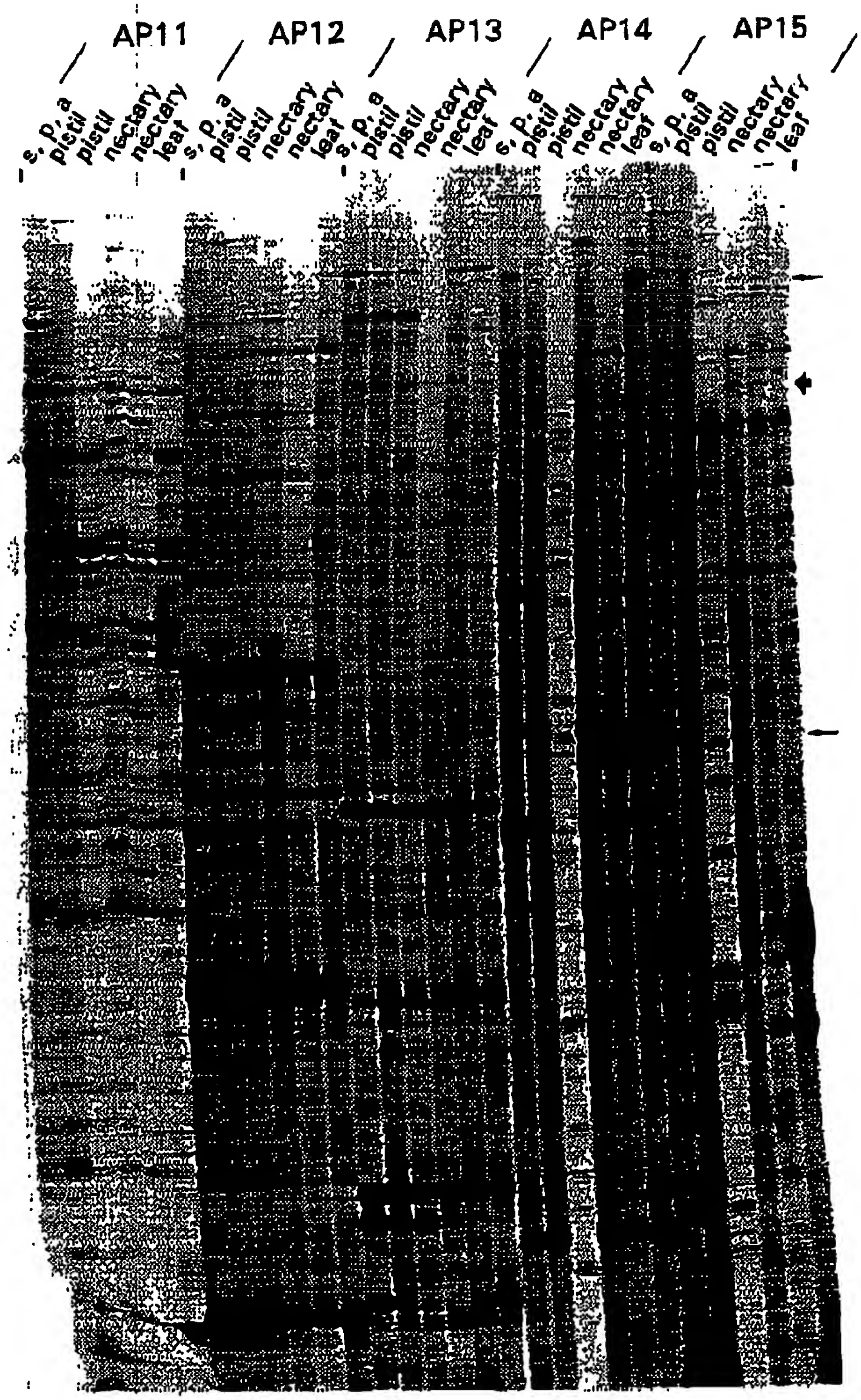

FIG.1

```
1     TGATCCTGTT CGAGAAGCTA CAAAATCAAA AGAAGGTGTA GAAATCATTA
51    TCAACATTGA AGATGATAAT TCTGATAACG CATTGCACTC CATGGAGAAG  prat 119 ←
101   GATTTTTCCA CACTCCCCAC ATCAAAATAA CCAAGAAGAT GATCAAAAAA
151   TGACAAAGCT AAGGAGTTTG AAGTAAGGCA AGGAACTTGA CACTGAATAT
201   CTAAGCTAAT TAGCAAGACT TTAGCAGCTT GTAATATTTA GTGTTTGTGA
251   GGTGTTACCT TATAATTAGC TTGTAGCATA GCCTTCCCAC TAATAATTCT  prat 122 ←
301   GCTTAGCGAA TCTTATATAT GGGAAATACT TACACTAGTA TGCATCTTCT
351   ATATACATGT TTGGCACTTG ACTATACATA GAAAAATTAA CAAGCATTTC
401   TCACCTCAAT TTGTCACTTA CTTATAAGTA GCTGAATAAT ATAATGCAAT
451   TTTCACCCC
```

FIG. 2

```
1    TCGAGCGGCC GCCCGGGCAG GTATTCAACA AGAGTATTCA CCACTTGAAC
51   TCAAAAGGGG CTTCACTAAA AAAAAATCAT GGCGCAATTA CGTGCTGATG prat 129 →
101  ACTTGTCTTT CATATTTGGC CTTCTTGGTA ATATTGTATC ATTCATGGTC
151  TTCCTAGCAC CCGTGCCAAC ATTTTACAAA ATATATAAAA GGAAATCATC
201  AGAAGGATAT CAAGCAATAC CATATATGGT AGCACTGTTC AGCGCCGGAC
251  TATTGCTATA TTATGCTTAT CTCAGGAAGA ATGCCTATCT TATCGTCACC
301  ATTAATGGCT TTGGATGTGC CATTGAATTA ACATATATCT CTCTGTTTCT
351  CTTTTAGGCG CCCAGAAAGT CTAAGATTTT CACAGGGTGG CTGATGCTCT
401  TAGAATTGGG AGCCCTAGGA ATCCTGATGC CAATTACTTA TTTATTAGCA
451  GAAGGCTCAC ATAGAGTGAT CATAGTGGCA TGGATTTGTG CAGCTATCAA
501  TGTTGCTGTC TTTGCTGCTC CTTTAAGCAT CATGAGGCAA GTAATAAAAA
551  CAAAGAGTGT AGAGTTCATG CCCTTCACTT TATCTTTGTT CCTCACTCTC
601  TGTGCCACTA TCTGGTTTTT CTATGGGTTT TTCAAGAAGG ACTTTTACAT
651  TGCGTTTCCA AATATACTGG GCTTTCTATT CGCAATCCTT CAAATGCTAT
701  TATATTTTGT TTACAAGGAT TCAAAGAGAA TAGATGATGA AAAATCTCAT
751  CCTGTTCGAG AAGCTACAAA ATCAAAAGAA GGTGTAGAAA TCATTATCAA
801  CATTGAAGAT GATAATTCTG ATAACGCATT GCAGTCCATG GAGAAGG
```

FIG.3

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1 TCGAGCGGCC GCCCGGGCAG GTATTCAACA AGACTATTCA CCACTTGAAC
51 TCAAAAGGGG CTTCACTAAA AAAAATCAT GGCGCAATTA CGTGCTGATG
101 ACTTGTCTTT CATATTTGGC CTTCTTGGTA ATATTGTATC ATTCATGGTC
151 TTCCTAGCAC CCGTGCCAAC ATTTTACAAA ATATATAAAA GGAAATCATC
201 AGAAGGATAT CAAGCAATAC CATATATGGT AGCACTGTTC AGCGCCGGAC
251 TATTGCTATA TTATGCTTAT CTCAGGAAGA ATCCCTATCT TATCGTCAGC
301 ATTAATGGCT TTGGATGTCC CATTGAATTA ACATATATCT CTCTGTTTCT
351 CTTTTACCCG CCCAGAAAGT CTAAGATTTT CACAGGGTGG CTGATGCTCT
401 TAGAATTGGG AGCCCTAGGA ATGGTGATGC CAATTACTTA TTTATTAGCA
451 GAAGGCTCAC ATAGAGTGAT GATAGTGGGA TGGATTTGTG CAGCTATCAA
501 TGTTGCTGTC TTTGCTGCTC CTTTAAGCAT CATGAGCCAA GTAATAAAAA
551 CAAACACTGT AGAGTTCATG CCCTTCACTT TATCTTTGTT CCTCACTCTC
601 TGTGCCACTA TGTGGTTTTT CTATGGGTTT TTCAAGAAGG ACTTTTACAT
651 TGCGTTTCCA AATATACTGG GCTTTCTATT CGGAATCGTT CAAATGCTAT
701 TATATTTTGT TTACAAGGAT TCAAAGAGAA TAGATGATCA AAAATCTGAT
751 CCTGTTCGAG AAGCTACAAA ATCAAAAGAA GGTGTAGAAA TCATTATCAA
801 CATTGAAGAT GATAATTCTG ATAACGCATT GCAGTCCATG GAGAAGGATT
851 TTTCCAGACT GCGGACATCA AAATAAGCAA GAAGATGATC AAAAAATGAC
901 AAAGCTAAGG AGTTTGAAGT AAGGCAAGGA ACTTGACACT GAATATCTAA
951 GCTAATTAGC AAGACTTTAC CACCTTGTAA TATTTAGTGT TTGTGAGGTG
1001 TTACCTTATA ATTAGCTTCT AGCATAGCCT TCCCACTAAT AATTCTGCTT
1051 AGCGAATCTT ATATATGGGA AATACTTACA CTAGTATGCA TCTTCTATAT
1101 ACATGTTTGG CACTTGACTA TACATAGAAA AATTAACAAG CATTTCTCAC
1151 CTCAATTTGT CACTTACTTA TAAGTACCTC AATAATATAA TGCAATTTTC
1201 ACCCC

FIG. 4

09/743885

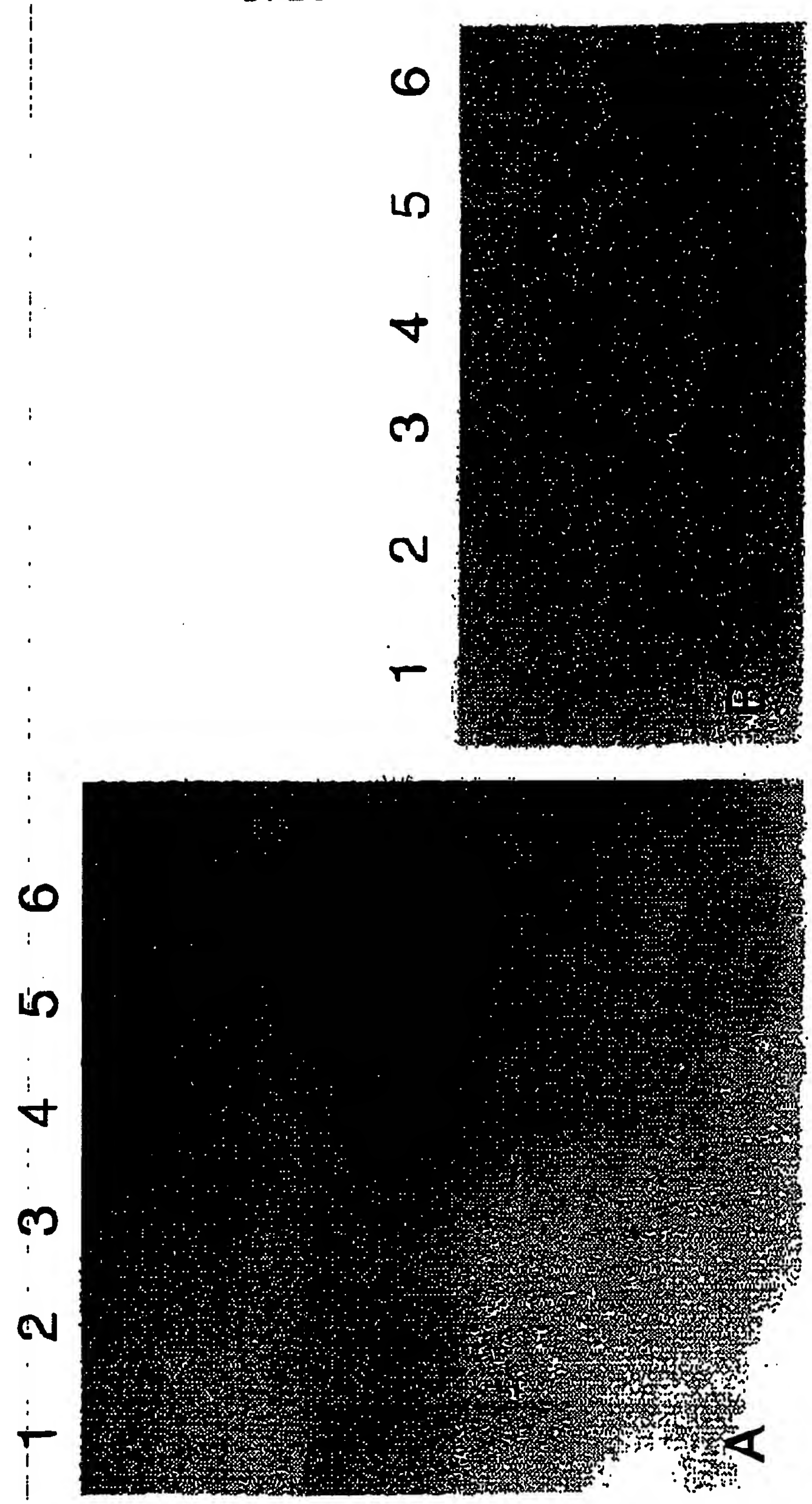

FIG.5

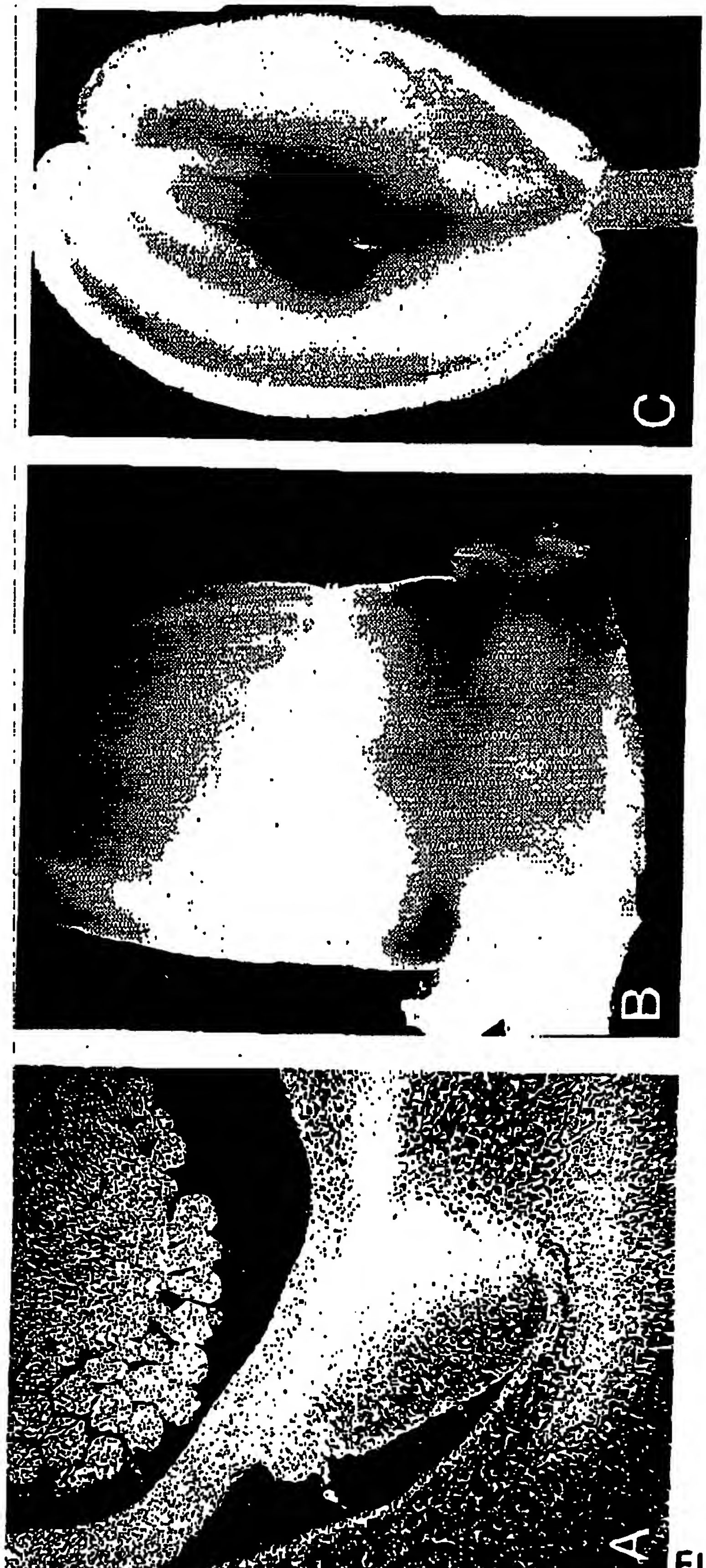

FIG.6

1 CCTAGGAGAAATCAAGCCTACTCTTAAGATGGATGACTCACTTGCCCCGA
51 TGGTAAGGTGAACCATCTGTTGATTAGAGTTGGAAGTTCATGTTCTCTG
101 CTGATTTTATTATTCTAGACTATGAAGAGGACCAAGAAGCTCCAATAATT
151 TTGGAAGAGCATTCTTAATCACATCGATGGCAATTATTGACATGGAACT
201 TGGGGAGATGACTGTGAGACCCCATGGAGAAAAGGTTACTTTCAAGGTTT
251 ATAATAAAAAGGATCATATGGCTAAGTTTGAAGAGTGTTCTTTGATAGAA
301 TGTGTCAGACGAGAACATGAAAGTAAACCGAAAGAGGTGTTTGAGCGGAA
351 TGTAGAACAAAGTGACCACGGCACAATAATTGACAAGTTGAAGGAAAATT
401 CACCTAAAGGAAGGAAGAAGACAAAAGTTCGTCGTAACAAGAGGAGACCT
451 AAATGCTGGAAGTGAGCTTAAAGGTGTTGTCGTACTACGACGTTAACTAA
501 GGCGCTTGTCGGGAGGCAACCCTAGCTTTCTATGTAAATGTAAAAGTAAA
551 AAATATATATATAGAAAAAGGAAAATACAAAAAGAGTCGTGCCCCGACGT
601 TAAATCAAGCGCTTGTTGGAAGGCAACCCAATTTTTATTGTTTTAGTTGT
651 TTTACTTATTTAGTATTACCTAGTTTCTTGTTGTTTTTGTAGGGCTCGGG
701 ACTTTCGGAAGGTGAGGTAATTTCAAGGCATCGCGGTGTCTATTGCAGCG
751 AGGTAAGTGTAAGAGTTGAGTTGGAAGCGTTTGGCCAAGTGTTGCACCGT
801 GAGAGGCTTTCAACCTGTTGCGACACGTGAAAAATTAAGAGCCAGATCTG
851 CTACATTAGCACTGAAGCATCGCTTGGCCAATAGCTTGGAATGGAAGCAA
901 GAATTCAAACCAAAATCAGAAACGCCACAAGAGATGTGTCGCACACTCCA
951 AAGCTTTGTGCAAACTAGTGAACGCAGAAATAGAAATGCTACAGCCCATG
1001 CGTCGCTTGGCTTATGGCAGGCAGCAAAAATTCAGCACCAAAACAGAAAC
1051 GCTGCGAGAAACGCGTCGCATACGCCATAGCTTTGTGTCAAACACAACGT
1101 CCAGAAATTGAAAAGCTATAAGCCTGCGTCGCTTGGCTCATGGCGTGCAG
1151 ACTAGAAAAGCTCTAGCAGATCCCTCGCGTATTGTATAGCTTGGTGTGAA
1201 ACAGAAAGTTCGAAACTTGGAAAACGATAACCCAGCGTCGCCTCTTCAAC
1251 CGCGTCCAGGTAAGTTCAAGATTCTTACGGGTTGACCCATTAACCCATTG
1301 ATCGGCTGATTATAAACAATAAAACATCACCTTCAACTATCACATGATTT
1351 CATAAGTTTGACCTAGGATATTTTATATATATATATATATATATACACAC

FIG.7

1401 ACACACCATTTCCAGCGATCTTACCTCATTTTTATTCAAACCATTTTTCT

1451 GCTTCAAAAGTTTAAATTATTAATATGATAAGTCATCCATAGTCAAACAA

1501 GATTTTCTATACTATTTTGTCCCTTGTAATTTTAAAAAAAAAATGAGCGA

1551 TGGTAAGATAAACATTGTTTGCAAGTGTACAATTTTAGTATATGCAAACC

1601 AACGCTTCTTCTTCCAACTATCACCTAAAACTACATCATTTATGGCGGGC

1651 GGACTACACGTAGCCAAATATAAAAACGCAATGGCCATTCACTTCATGTC

1701 ATTTTTATATCCTTCATCCAATAATATTACTCAAAATTGATGTACAGTTT

1751 GGTCTCTGATGTGCACTTTACTATACGTAATACGGAATTTACATTATAAT

1801 TAAAGAGAACTGTTCCACTAAATTTTAATGATTTAATTAATTTAACTCGG

1851 TTACTTGTATTATTATTATTGCTCTATTTGTTTGTCATTTGAATTTGGCA

1901 CCGCAGATTTTTGTATGCAATTAACCCTCATATATCTTTTGGCCAAATAA

1951 AGAAAAAGTCTGCATATTTCTTGCCAAACATTTATCATACTTTACCGAAT

2001 TCTTGTTTTTTGTTTCTCTGTTCTTGTTCTCCACTATAAATAACATTTGC

2051 AGTGAGTAAAGTTTCTTCAGGTCTCTTTTGTAGATTCAACAAGAGTATTC

2101 AGCACTTGAACTCAAAAGGGGCTTCACTAAAAAAAATCATG

FIG.7(CONTIN.)

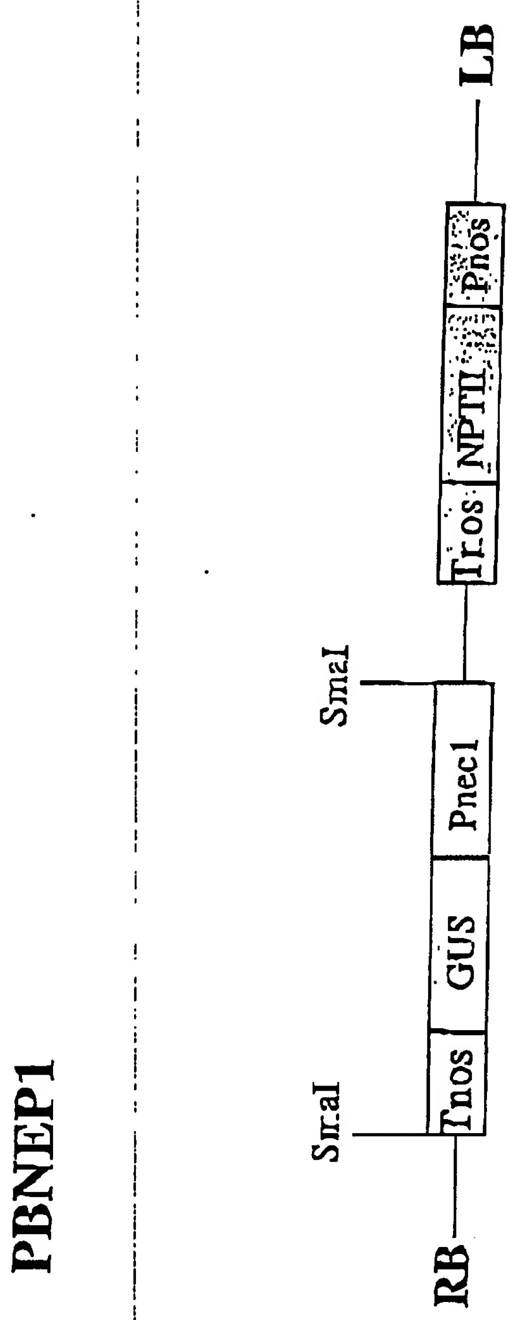

FIG. 8

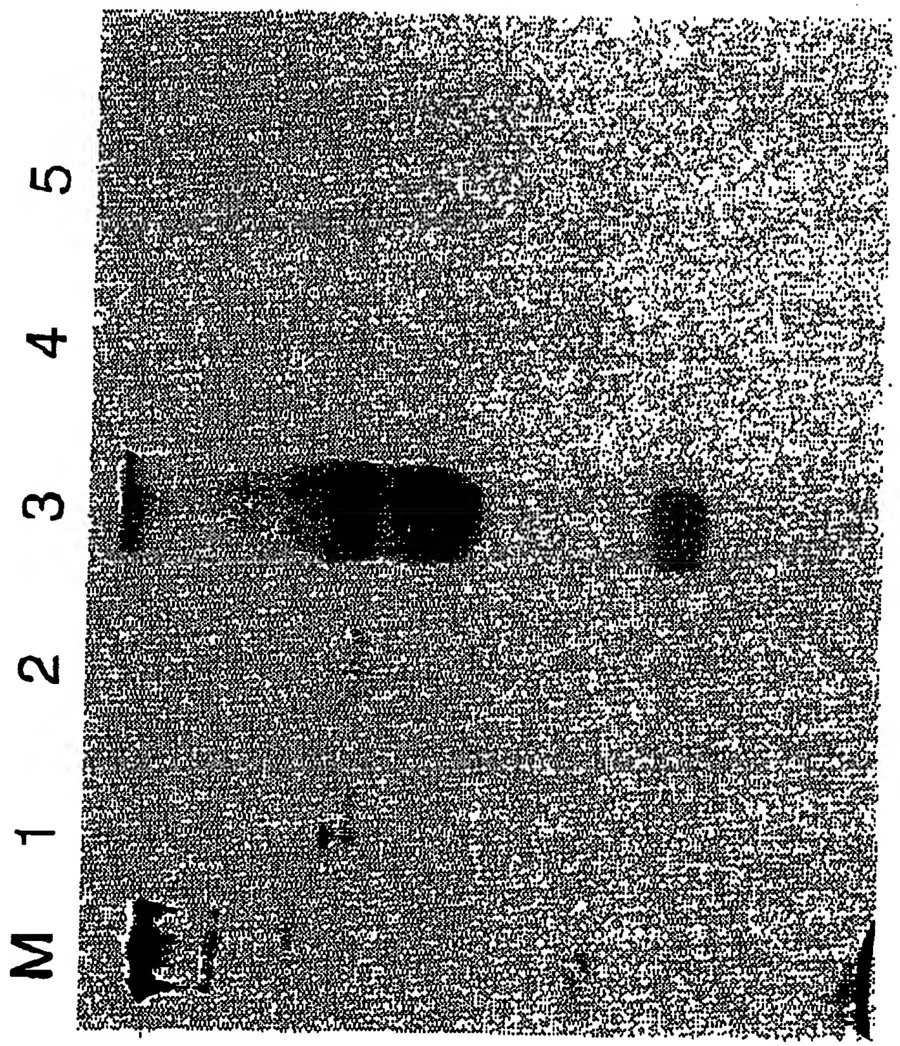

FIG. 9

09/743885

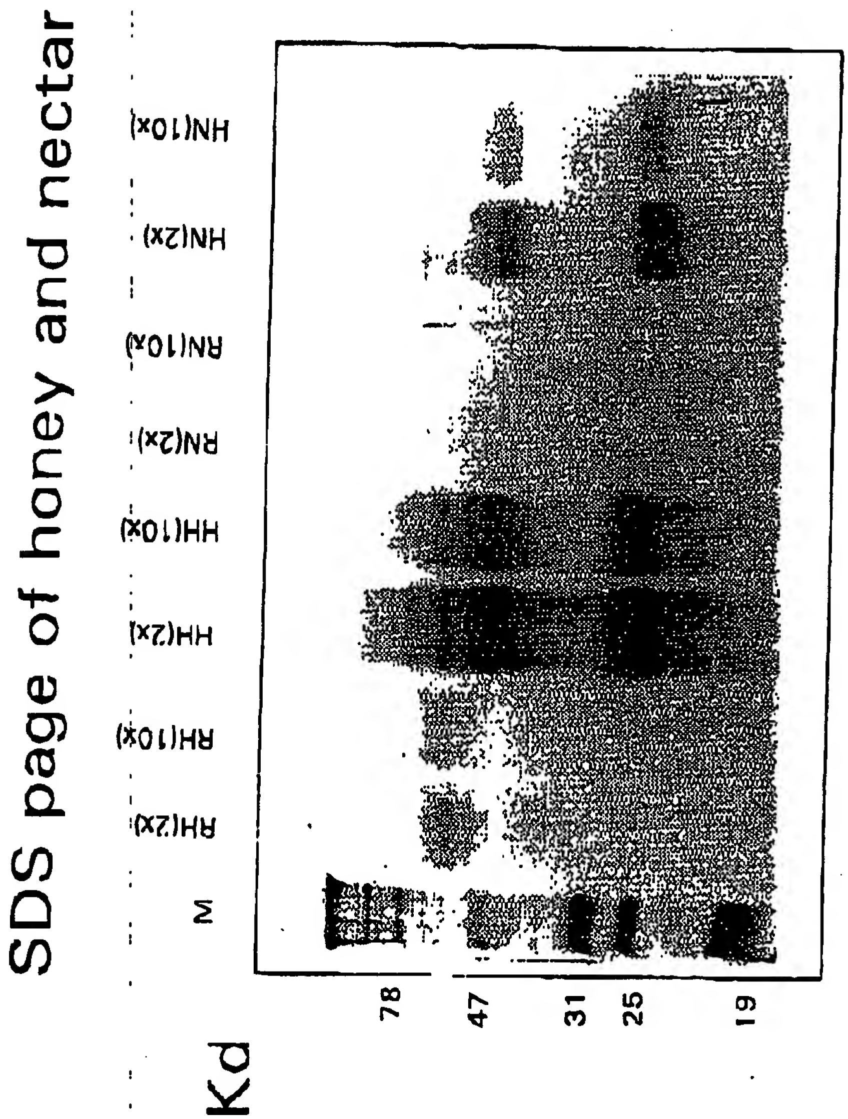

FIG.10

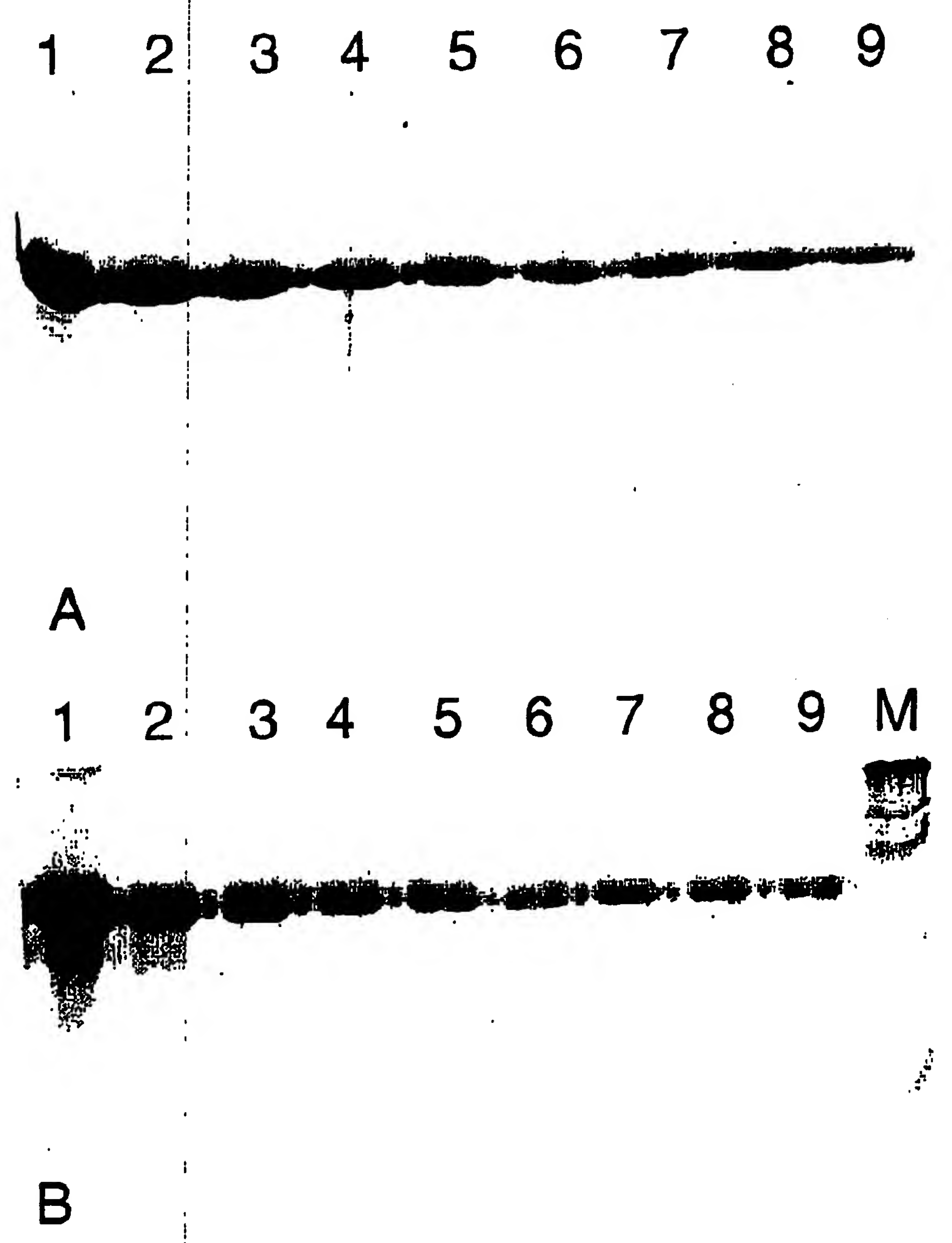

FIG.11

13/20

```
===17-APR-1998=================================================PC/GENE===

****************************************
* ALIGNMENT OF TWO PROTEIN SEQUENCES.  *
****************************************

The two sequences to be aligned are:

PCVH29.
Total number of residues: 60.

GER1.
Total number of residues: 211.

Comparison matrix  : Structure-genetic matrix
Open gap cost      : 7
Unit gap cost      : 1

The character to show that two aligned residues are identical is '|'

PCVH29     - MKMFLPILFTISLLFSSSHASVLDFCVADPSLPDGPACYSCKEPAKVTVD -50
             |||  . | |   ||  | | ||| |||||||   |   | ||||| |  ||
GER1       - MKMRIQIFFILSLFSSISPASVQDFCVADPKGPQNPSGYSCKNPDQVTEN -50

PCVH29     - DFVFRGLGTA                                         -60
             || | |||| |
GER1       - DFAFSGLGKAGNTSNVIKAAVTPAPAPAFAGLNGLDVSLARLDLACGGVI -100

GER1       - PLHTHPGASEVLVVIQCTICAGFISSANKVYLKTLSRGDSNVPPQGLLHF -150

GER1       - QLNSGKGPALAFVAFGSSSPGLQILPFALFANDLPSELVEATTFLSDEEV -200

GER1       - KKLKGVLGGTN -211

Identity   : 36 ( 60%)
Number of gaps inserted in PCVH29: 0
Number of gaps inserted in GER1: 0

---17-APR-1998=================================================PC/GENE---
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FIG.12

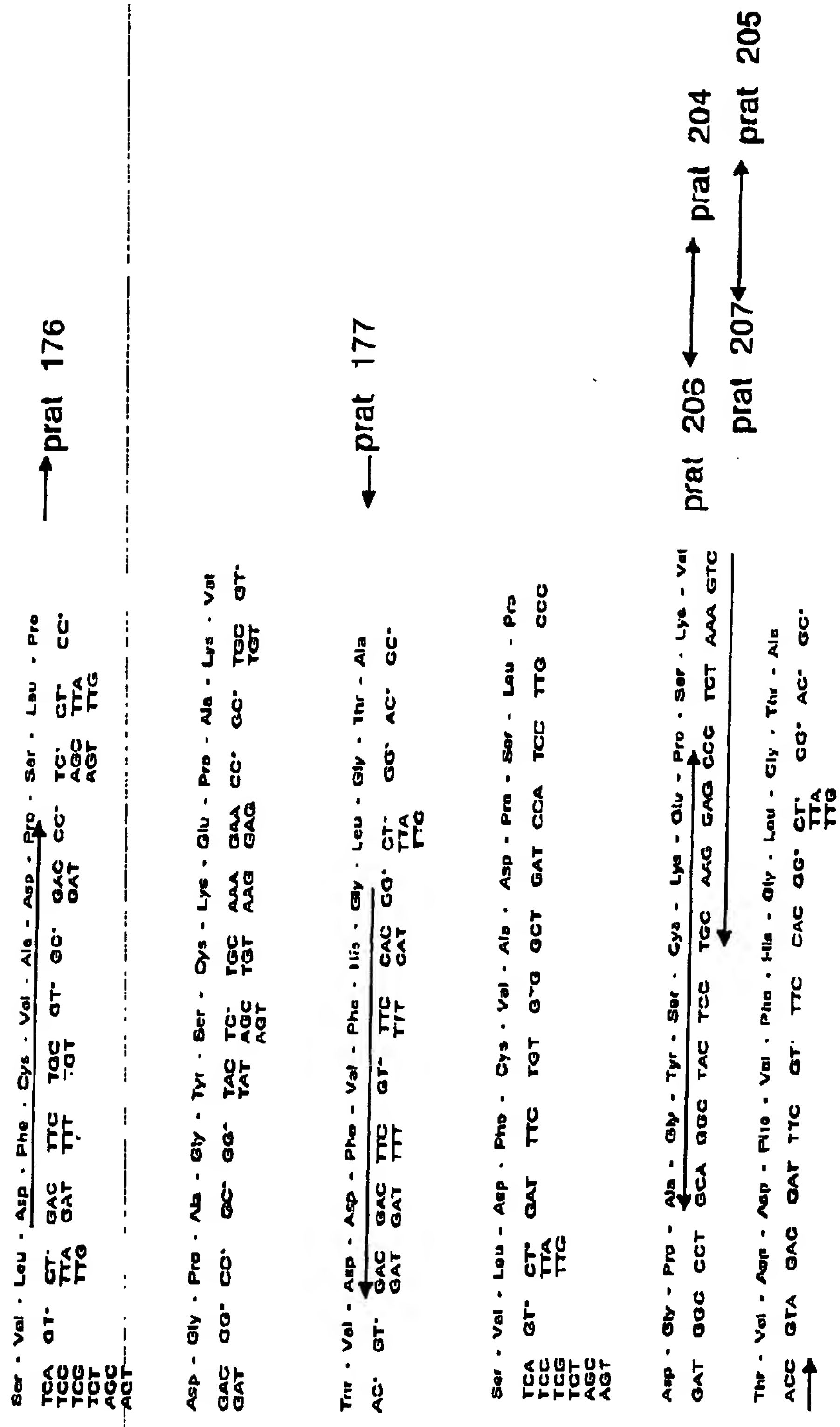

FIG.13

```
MAR5H6   CGCCCGGGCTGGTAAACAAAGTACGTGTATACTCATTGTATTTCCTTAAA   50
MAR5R8   CGCCCGGGCTGGTAAACAAAGTACGTGTATACTCATTGTATTTCCTTAAA   50
MAR5R5   GGCCCGGGCTGGTAAACAAAGTACGTGTATACTCATTGTATTTCCTTAAA   50
MAR5H8   CGCCCGGGCTGGTAAACAAAGTACGTGTATACTCATTGTATTTCCTTAAA   50
          *************************************************

MAR5H6   AAAGCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC   100
MAR5R8   AAAGCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC   100
MAR5R6   AAAGCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC   100
MAR5H8   AA-GCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC   99
         ** ***********************************************

MAR5H6   CCCTATTGGACAACACGAACTCTAAGAGGGCAATCAGACACACCAGCCAT   150
MAR5R8   CCCTATTGGACAACACGAACTCTAAGAGGGCAATCAGACACACCAGCCAT   150
MAR5R6   CCCTATTGGACAACACGAACTCTAAGAGGGCAATCAGACACACCAGCCAT   150
MAR5H8   CCCTATTAAACACCACGAACTCTAAGAGGGCAATCAGACACACCAGCCAT   149
         *******..*** *************************************

MAR5H6   TGCACTTGTGAAGATGTTTCTTCCAATTCTCTTCACCATTTCCCTCCTCT   200
MAR5R8   TGCACTTGTGAAGATGTTTCTTCCAATTCTCTTCACCATTTCCCTCCTCT   200
MAR5R6   TGCACTTGTGAAGATGTTTCTTCCAATTCTCTTCACCATTTCCCTCCTCT   200
MAR5H8   TGCACTTATGAAGATGTTTCTTCCAATTCTCTTCACCATTTCCCTCCTCT   199
         *******.******************************************

         signal sequence <--|--> mature protein
MAR5H6   TCTCCTCCTCCCATGCTTCAGTGTTGGACTTCTGCGTAGCAGACCCATCC   250
MAR5R8   TCTCCTCCTCCCATGCTTCAGTGTTGGACTTCTGCGTAGCAGACCCATCC   250
MAR5R6   TCTCCTCCTCCCATGCTTCAGTGTTGGACTTCTGCGTAGCAGACCCATCC   250
MAR5H8   TCTCCTCCTCCCATGCTTCAGTGTTGGACTTCTGCGTAGCAGGCCCACCC   249
         ******************************************.**** **
```

FIG. 14

09/743885
528 Rec'd PCT/PTO 16 JAN 2001


```
tatgttcccttcgaattcctttcactatttctcttcttttctcttcttctcatgcatctgttcttgatttcg
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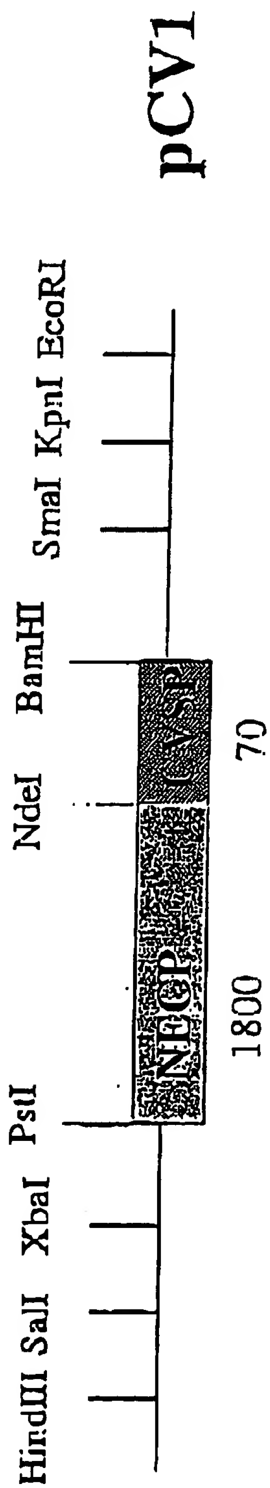
FIG.15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EcoRI
KpnI
SmaI
BamHI
NdeI
PstI
XbaI
SalI
HindIII
CVSP
NECP
70
1800

FIG.16

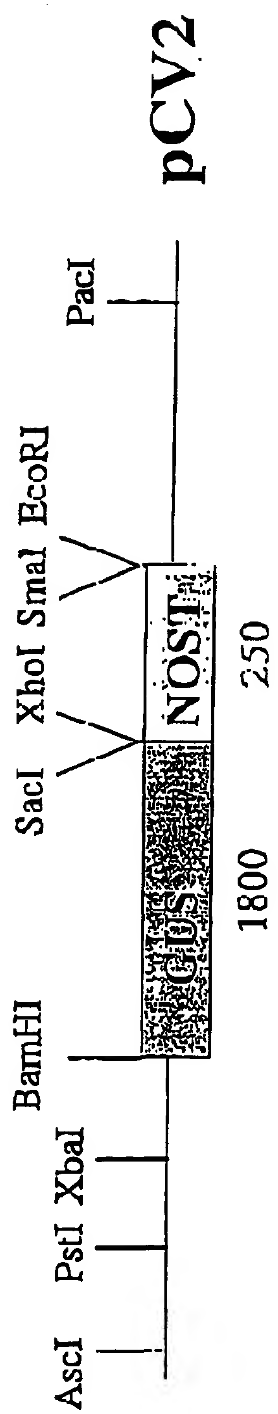
pCV2
PacI
EcoRI
SmaI
XhoI
SacI
NOST
250
GUS
1800
BamHI
XbaI
PstI
AscI

FIG.17

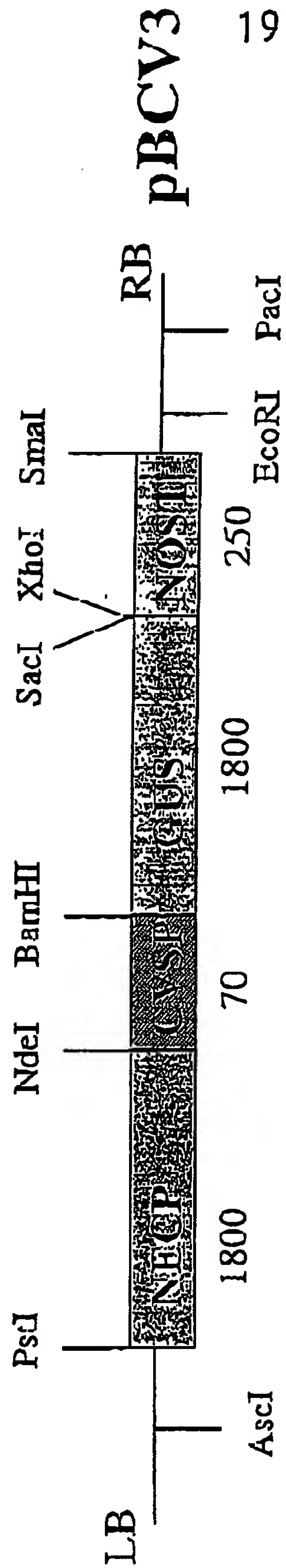
pBCV3
RB
PacI
EcoRI
SmaI
NOS
250
XhoI
SacI
GUS
1800
BamHI
70
NdeI
1800
PstI
AscI
LB

FIG. 18

09/743885

1 TCTGAATACAAGCTGTGTGTGTAGAGAGATTTCATAAAGACAGCAAACAT
51 CCCTTCTTTTTGTTCTGTTTTAAAAGTTCCCTTCTTCAACCAGCTCTTTT
101 CCTCATCAGGGTAAGTTGCAAATAAAGGGGATGTTCCAGAATCAAGAAGA
151 GAAGATGTCAGACTCGCCTCAGAGGAAGATGGGAAGAGGAAAGATTGAGA
201 TTAAGAGGATTCAAAATACAACAAATCGTCAAGTCACTTTCTGTAAGAGA
251 AGAAATGGGTTGCTTAAAAAAGCTTATGAACTTTCTGTTCTTTGTGATGC MADS-box
301 TGAAGTTGCTCTCATCGTTTTCTCAAGCCGTGGCCCCCTCTATGAATATG
351 CTAACAACAGTGTGAAGGCAACAATTGATAGATATAAGAAAGCATCCTCA
401 GATTCCTCCAACACTGGATCTACTTCTGAAGCTAACACTCAGTTTTATCA
451 ACAAGAAGCTGCCAAACTCCGAGTTCAGATTGGTAACTTACAGAACTCAA
501 ACAGGAACATGCTAGGCGAGTCTCTAAGTTCTCTGACTGCAAAAGATCTG
551 AAAGGCCTGGACACCAAACTTGAGAAAGGAATTAGTAGAATTAGGTCCAA K-box
601 AAAGAATCAACTCCTGTTTCCTGAGATTGAGTATATGCGAAAAAGGGAAA
651 TTGATTTGCACAACAACAATCAGATGCTTCGGGCAAAGATAGCTGAGAGT
701 GAAAGAAATGTGAACATGATGGGAGGAGAATTTGAGCTGATGCAATCTCA
751 TCCGTACGATCCAAGAGACTTCTTCCAAGTGAACGGCTTACACCATAATC
801 ATCAATATCCACGCCAAGACAACATGGCTCTTCAATTAGTATAAGTTTAT
851 AATAAAATGCATGGTTTGAAGCACTCTGATTGTGGTGGATTTGGATTATG
901 TATAAGGCACTGCAGGCCATTTGCCAATTATTGAAAGGTACTCAAACAGG
951 AAGTTGAAGAAGTTCATCATCTCTCTCATCTATATGTCTTAACAAAAGTC
1001 TTAGCTTATGGACTCTAAAACAAAGACTTAATTTAACATATAAATATAAT
1051 TGTGTAATGCTGTTGTATTGTATGGTATCTATCCAAAAACATTAATAACC
1101 TATCTTTTTCTTCAAATTATGTCTCCTTTGATACAAACTACTAACATATT
1151 TTCTTAT

FIG.19

09/743885
528 Rec'd PCT/PTO 16 JAN 2001

SEQUENCE LISTING

<110> CPRO-DLO

<120> Process to collect metabolites from modified nectar by insects

<130> 159782

<140> pct/nl99/00453
<141> 1999-07-15

<160> 10

<170> PatentIn Ver. 2.1

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<212> PRT
<213> Petunia x hybrida

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<220>
<223> tissue type: nectar gland

<220>
<223> NEC1 amino acid sequence

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Met Ala Gln Leu Arg Ala Asp Asp Leu Ser Phe Ile Phe Gly Leu Leu
1 5 10 15

Gly Asn Ile Val Ser Phe Met Val Phe Leu Ala Pro Val Pro Thr Phe
20 25 30

Tyr Lys Ile Tyr Lys Arg Lys Ser Ser Glu Gly Tyr Gln Ala Ile Pro
35 40 45

Tyr Met Val Ala Leu Phe Ser Ala Gly Leu Leu Leu Tyr Tyr Ala Tyr
50 55 60

Leu Arg Lys Asn Ala Tyr Leu Ile Val Ser Ile Asn Gly Phe Gly Cys
65 70 75 80

Ala Ile Glu Leu Thr Tyr Ile Ser Leu Phe Leu Phe Tyr Ala Pro Arg
85 90 95

Lys Ser Lys Ile Phe Thr Gly Trp Leu Met Leu Leu Glu Leu Gly Ala
100 105 110

Leu Gly Met Val Met Pro Ile Thr Tyr Leu Leu Ala Glu Gly Ser His
115 120 125

Arg Val Met Ile Val Gly Trp Ile Cys Ala Ala Ile Asn Val Ala Val
130 135 140

Phe Ala Ala Pro Leu Ser Ile Met Arg Gln Val Ile Lys Thr Lys Ser
145 150 155 160

Val Glu Phe Met Pro Phe Thr Leu Ser Leu Phe Leu Thr Leu Cys Ala
165 170 175

Thr Met Trp Phe Phe Tyr Gly Phe Phe Lys Lys Asp Phe Tyr Ile Ala
180 185 190

Phe Pro Asn Ile Leu Gly Phe Leu Phe Gly Ile Val Gln Met Leu Leu
195 200 205

Tyr Phe Val Tyr Lys Asp Ser Lys Arg Ile Asp Asp Glu Lys Ser Asp
210 215 220

Pro Val Arg Glu Ala Thr Lys Ser Lys Glu Gly Val Glu Ile Ile Ile
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Asn Ile Glu Asp Asp Asn Ser Asp Asn Ala Leu Gln Ser Met Glu Lys
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Asp Phe Ser Arg Leu Arg Thr Ser Lys
260 265

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<213> Petunia x hybrida

<220>
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<223> tissue type: nectar gland, secretory cell

<220>

<223> FBP15 amino acid sequence

<400> 2

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Met Gly Arg Gly Lys Ile Gln Ile Lys Arg Ile Glu Asn Thr Thr Asn
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Arg Gln Val Thr Phe Cys Lys Arg Arg Asn Gly Leu Leu Lys Lys Ala
            20                  25                  30

Tyr Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Leu Ile Val Phe
        35                  40                  45

Ser Ser Arg Gly Arg Leu Tyr Glu Tyr Ala Asn Asn Ser Val Lys Ala
    50                  55                  60

Thr Ile Asp Arg Tyr Lys Lys Ala Ser Ser Asp Ser Ser Asn Thr Gly
65                  70                  75                  80

Ser Thr Ser Glu Ala Asn Thr Gln Phe Tyr Gln Gln Glu Ala Ala Lys
                85                  90                  95

Leu Arg Val Gln Ile Gly Asn Leu Gln Asn Ser Asn Arg Asn Met Leu
            100                 105                 110

Gly Glu Ser Leu Ser Ser Leu Thr Ala Lys Asp Leu Lys Gly Leu Glu
        115                 120                 125

Thr Lys Leu Glu Lys Gly Ile Ser Arg Ile Arg Ser Lys Lys Asn Glu
    130                 135                 140

Leu Leu Phe Ala Glu Ile Glu Tyr Met Arg Lys Arg Glu Ile Asp Leu
145                 150                 155                 160

His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
                165                 170                 175

Asn Val Asn Met Met Gly Gly Glu Phe Glu Leu Met Gln Ser His Pro
            180                 185                 190

Tyr Asp Pro Arg Asp Phe Phe Gln Val Asn Gly Leu Gln His Asn His
        195                 200                 205

Gln Tyr Pro Arg Gln Asp Asn Met Ala Leu Gln Leu Val
    210                 215                 220
```

<210> 3

<211> 18
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<213> Calluna vulgaris

<220>
<223> tissue type: flower

<220>
<223> Calluna vulgaris signal peptide

<400> 3
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1 5 10 15

His Ala

<210> 4
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<212> DNA
<213> Petunia x hybrida

<220>
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<222> (79)..(873)

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<220>
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cttcactaaa aaaaaatc atg gcg caa tta cgt gct gat gac ttg tct ttc 111
Met Ala Gln Leu Arg Ala Asp Asp Leu Ser Phe
1 5 10

ata ttt ggc ctt ctt ggt aat att gta tca ttc atg gtc ttc cta gca 159
Ile Phe Gly Leu Leu Gly Asn Ile Val Ser Phe Met Val Phe Leu Ala
15 20 25

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ccc gtg cca aca ttt tac aaa ata tat aaa agg aaa tca tca gaa gga    207
Pro Val Pro Thr Phe Tyr Lys Ile Tyr Lys Arg Lys Ser Ser Glu Gly
        30                  35                  40

tat caa gca atc cca tat atg gta gca ctg ttc agc gcc gga cta ttg    255
Tyr Gln Ala Ile Pro Tyr Met Val Ala Leu Phe Ser Ala Gly Leu Leu
    45                  50                  55

cta tat tat gct tat ctc agg aag aat gcc tat ctt atc gtc agc att    303
Leu Tyr Tyr Ala Tyr Leu Arg Lys Asn Ala Tyr Leu Ile Val Ser Ile
60                  65                  70                  75

aat ggc ttt gga tgt gcc att gaa tta aca tat atc tct ctg ttt ctc    351
Asn Gly Phe Gly Cys Ala Ile Glu Leu Thr Tyr Ile Ser Leu Phe Leu
                80                  85                  90

ttt tac gcg ccc aga aag tct aag att ttc aca ggg tgg ctg atg ctc    399
Phe Tyr Ala Pro Arg Lys Ser Lys Ile Phe Thr Gly Trp Leu Met Leu
            95                  100                 105

tta gaa ttg gga gcc cta gga atg gtg atg cca att act tat tta tta    447
Leu Glu Leu Gly Ala Leu Gly Met Val Met Pro Ile Thr Tyr Leu Leu
        110                 115                 120

gca gaa ggc tca cat aga gtg atg ata gtg gga tgg att tgt gca gct    495
Ala Glu Gly Ser His Arg Val Met Ile Val Gly Trp Ile Cys Ala Ala
    125                 130                 135

atc aat gtt gct gtc ttt gct gct cct tta agc atc atg agg caa gta    543
Ile Asn Val Ala Val Phe Ala Ala Pro Leu Ser Ile Met Arg Gln Val
140                 145                 150                 155

ata aaa aca aag agt gta gag ttc atg ccc ttc act tta tct ttg ttc    591
Ile Lys Thr Lys Ser Val Glu Phe Met Pro Phe Thr Leu Ser Leu Phe
                160                 165                 170

ctc act ctc tgt gcc act atg tgg ttt ttc tat ggg ttt ttc aag aag    639
Leu Thr Leu Cys Ala Thr Met Trp Phe Phe Tyr Gly Phe Phe Lys Lys
            175                 180                 185

gac ttt tac att gcg ttt cca aat ata ctg ggc ttt cta ttc gga atc    687
Asp Phe Tyr Ile Ala Phe Pro Asn Ile Leu Gly Phe Leu Phe Gly Ile
        190                 195                 200

gtt caa atg cta tta tat ttt gtt tac aag gat tca aag aga ata gat    735
Val Gln Met Leu Leu Tyr Phe Val Tyr Lys Asp Ser Lys Arg Ile Asp
    205                 210                 215
```

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gat gaa aaa tct gat cct gtt cga gaa gct aca aaa tca aaa gaa ggt    783
Asp Glu Lys Ser Asp Pro Val Arg Glu Ala Thr Lys Ser Lys Glu Gly
220                 225                 230                 235

gta gaa atc att atc aac att gaa gat gat aat tct gat aac gca ttg    831
Val Glu Ile Ile Ile Asn Ile Glu Asp Asp Asn Ser Asp Asn Ala Leu
                240                 245                 250

cag tcc atg gag aag gat ttt tcc aga ctg cgg aca tca aaa            873
Gln Ser Met Glu Lys Asp Phe Ser Arg Leu Arg Thr Ser Lys
            255                 260                 265

taagcaagaa gatgatcaaa aaatgacaaa gctaaggagt ttgaagtaag gcaaggaact 933

tgacactgaa tatctaagct aattagcaag actttagcag cttgtaatat ttagtgtttg 993

tgaggtgtta cctcataatt agcttgtagc atagccttcc cactaataat tctgcttagc 1053

gaatcttata tatgggaaat acttacacta gtatgcatct tctatataca tgtttggcac 1113

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Gly Asn Ile Val Ser Phe Met Val Phe Leu Ala Pro Val Pro Thr Phe
             20                  25                  30

Tyr Lys Ile Tyr Lys Arg Lys Ser Ser Glu Gly Tyr Gln Ala Ile Pro
         35                  40                  45

Tyr Met Val Ala Leu Phe Ser Ala Gly Leu Leu Leu Tyr Tyr Ala Tyr
     50                  55                  60

Leu Arg Lys Asn Ala Tyr Leu Ile Val Ser Ile Asn Gly Phe Gly Cys
 65                  70                  75                  80

Ala Ile Glu Leu Thr Tyr Ile Ser Leu Phe Leu Phe Tyr Ala Pro Arg
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85 90 95

Lys Ser Lys Ile Phe Thr Gly Trp Leu Met Leu Leu Glu Leu Gly Ala
100 105 110

Leu Gly Met Val Met Pro Ile Thr Tyr Leu Leu Ala Glu Gly Ser His
115 120 125

Arg Val Met Ile Val Gly Trp Ile Cys Ala Ala Ile Asn Val Ala Val
130 135 140

Phe Ala Ala Pro Leu Ser Ile Met Arg Gln Val Ile Lys Thr Lys Ser
145 150 155 160

Val Glu Phe Met Pro Phe Thr Leu Ser Leu Phe Leu Thr Leu Cys Ala
165 170 175

Thr Met Trp Phe Phe Tyr Gly Phe Phe Lys Lys Asp Phe Tyr Ile Ala
180 185 190

Phe Pro Asn Ile Leu Gly Phe Leu Phe Gly Ile Val Gln Met Leu Leu
195 200 205

Tyr Phe Val Tyr Lys Asp Ser Lys Arg Ile Asp Asp Glu Lys Ser Asp
210 215 220

Pro Val Arg Glu Ala Thr Lys Ser Lys Glu Gly Val Glu Ile Ile Ile
225 230 235 240

Asn Ile Glu Asp Asp Asn Ser Asp Asn Ala Leu Gln Ser Met Glu Lys
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Asp Phe Ser Arg Leu Arg Thr Ser Lys
260 265

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flowers

<220>
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aataaagggg atgttccaga atcaagaaga gaagatgtca gactcgcctc agaggaag 178

atg gga aga gga aag att gag att aag agg att gaa aat aca aca aat 226
Met Gly Arg Gly Lys Ile Glu Ile Lys Arg Ile Glu Asn Thr Thr Asn
1 5 10 15

cgt caa gtc act ttc tgt aag aga aga aat ggg ttg ctt aaa aaa gct 274
Arg Gln Val Thr Phe Cys Lys Arg Arg Asn Gly Leu Leu Lys Lys Ala
20 25 30

tat gaa ctt tct gtt ctt tgt gat gct gaa gtt gct ctc atc gtt ttc 322
Tyr Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Leu Ile Val Phe
35 40 45

tca agc cgt ggc cgc ctc tat gaa tat gct aac aac agt gtg aag gca 370
Ser Ser Arg Gly Arg Leu Tyr Glu Tyr Ala Asn Asn Ser Val Lys Ala
50 55 60

aca att gat aga tat aag aaa gca tcc tca gat tcc tcc aac act gga 418
Thr Ile Asp Arg Tyr Lys Lys Ala Ser Ser Asp Ser Ser Asn Thr Gly
65 70 75 80

tct act tct gaa gct aac act cag ttt tat caa caa gaa gct gcc aaa 466
Ser Thr Ser Glu Ala Asn Thr Gln Phe Tyr Gln Gln Glu Ala Ala Lys
85 90 95

ctc cga gtc cag att ggt aac tta cag aac tca aac agg aac atg cta 514
Leu Arg Val Gln Ile Gly Asn Leu Gln Asn Ser Asn Arg Asn Met Leu
100 105 110

ggc gag tct cta agt tct ctg act gca aaa gat ctg aaa ggc ctg gag 562
Gly Glu Ser Leu Ser Ser Leu Thr Ala Lys Asp Leu Lys Gly Leu Glu

115 120 125

acc aaa ctt gag aaa gga att agt aga att agg tcc aaa aag aat gaa 610
Thr Lys Leu Glu Lys Gly Ile Ser Arg Ile Arg Ser Lys Lys Asn Glu
130 135 140

ctc ctg ttt gct gag att gag tat atg cga aaa agg gaa att gat ttg 658
Leu Leu Phe Ala Glu Ile Glu Tyr Met Arg Lys Arg Glu Ile Asp Leu
145 150 155 160

cac aac aac aat cag atg ctt cgg gca aag ata gct gag agt gaa aga 706
His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
165 170 175

aat gtg aac atg atg gga gga gaa ttt gag ctg atg caa tct cat ccg 754
Asn Val Asn Met Met Gly Gly Glu Phe Glu Leu Met Gln Ser His Pro
180 185 190

tac gat cca aga gac ttc ttc caa gtg aac ggc tta cag cat aat cat 802
Tyr Asp Pro Arg Asp Phe Phe Gln Val Asn Gly Leu Gln His Asn His
195 200 205

caa tat cca cgc caa gac aac atg gct ctt caa tta gta taagtttata 851
Gln Tyr Pro Arg Gln Asp Asn Met Ala Leu Gln Leu Val
210 215 220

ataaaatgca tggtttgaag cactctgatt gtggtggatt tggattatgt ataagggagt 911

gcaggccatt tgccaattat tgaaaggtac tcaaacagga agttgaagaa gttcatcatc 971

tctctcatct atatgtctta acaaaagtct tagcttatgg actctaaaac aaagacttaa 1031

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35 40 45

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Leu Arg Val Gln Ile Gly Asn Leu Gln Asn Ser Asn Arg Asn Met Leu
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Gly Glu Ser Leu Ser Ser Leu Thr Ala Lys Asp Leu Lys Gly Leu Glu
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His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 99/00453

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N5/10 C07K14/415 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KATER, M.M., ET AL.: "multiple AGAMOUS homologs from cucumber and petunia differ in their ability to induce reproductive organ fate" THE PLANT CELL, vol. 10, February 1998 (1998-02), pages 171-182, XP002094414 abstract, page 172, 182, Fig. 1,2 and 3c	11,12, 14,15
X	TSUCHIMOTO, S., ET AL.: "ectopic expression of pMADS3 in transgenic petunia phenocopies the petunia blind mutant" THE PLANT CELL, vol. 5, August 1993 (1993-08), pages 843-853, XP002094415 abstact, Fig. 2	11,12, 14,15

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 1999	05/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hultorf, S

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 99/00453

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANGENENT, G.C., ET AL.: "petal and stamen formation in petunia is regulated by the homeotic gene fbp1" THE PLANT JOURNAL, vol. 4, no. 1, 1993, pages 101-112, XP002094416 abstract; Fig. 8	11,12, 14,15
X	WILKINSON, J., ET AL.: "pollen as a vector in the dispersal of transgene products" JOURNAL OF EXPERIMENTAL BOTANY, vol. 45, 1994, page 42 XP002094992 the whole document	29,36,37
X	GOODALL, I., ET AL.: "contribution of high-performance liquid chromatographic analysis of carbohydrates to authenticity testing of honey" JOURNAL OF CHROMATOGRAPHY, vol. 706, 1995, pages 353-359, XP004038886 the whole document	29
A	THOMA, S., ET AL.: "tissue specific expression of a gene encoding a cell wall-localized lipid transfer protein from Arabidopsis" PLANT PHYSIOLOGY, vol. 105, 1994, pages 35-45, XP002094417 Fig. 6n; Fig. 7h; page 44, right column	1-37
A	TANG, X., ET AL.: "pistil-specific and ethylene-regulated expression of 1-aminocycopropane-1-carboxylate oxidase genes in petunia flowers" THE PLANT CELL, vol. 6, 1994, pages 1227-1239, XP002094418 abstract; Fig. 6e; page 1237	1-37
A	NEWMAN, T., ET AL.: "genes galore: a summary of methods for assessing results from large-scale partial sequencing of anonymous Arabidopsis cDNA clones" EMBL SEQUENCE DATA LIBRARY, 20 January 1996 (1996-01-20), XP002094419 heidelberg, germany accession no. N37251	2,7,8

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 99/00453

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	NEWMAN, T., ET AL.: "genes galore: a summary of methods for assessing results from large-scale partial sequencing of anonymous Arabidopsis cDNA clones" EMBL SEQUENCE DATA LIBRARY, 3 February 1995 (1995-02-03), XP002094420 heidelberg, germany accession no. T45181 -----	2,7,8

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 99/00453

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 6
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
   See additional sheet

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/NL 99/00453

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 6

Claim 6 refers to a SEQID No.8 that would - under the original nomenclature - represent the promoter of clone FBP15; a sequence is not given in the sequence listings. Accordingly, a reasonable search could not be performed for claim 6.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

PATENT COOPERATION TREATY

ONTVANGEN
27 NOV. 2000
BEANTWOORD

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

DE HOOP, Eric
Octrooibureau Vriesendorp & Gaade
Postbus 266
Dr. Kuyperstraat 6
2501 AW Den Haag
PAYS-BAS

Fax: +31 70 3646793

by fax and post

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing (day/month/year)	17. 11. 00	
Applicant's or agent's file reference 159/82	**IMPORTANT NOTIFICATION**	
International application No. PCT/NL 99/00453	International filing date (day/month/year) 15/07/1999	Priority date (day/month/year) 16/07/1998
Applicant CPRO-DLO et al.		

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx 523656 epmu d Fax: +49 89 2399 - 4465	Emslander, S Tel.+49 89 2399-8718

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 159782	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/NL99/00453	International filing date (day/month/year) 15/07/1999	Priority date (day/month/year) 16/07/1998
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant CPRO-DLO et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand 11/02/2000	Date of completion of this report 17. 11. 00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Schwachtgen, J-L Telephone No. +49 89 2399 8933

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL99/00453

I. **Basis of the report**

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).):*

Description, pages:

1-47 as originally filed

Claims, No.:

1-30 as received on 27/10/2000 with letter of 27/10/2000

Drawings, sheets:

1/20-20/20 as originally filed

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☒ the claims, Nos.: 31-38

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL99/00453

☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

see separate sheet

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-10, 12-15, 19, 20, 22, 24
	No:	Claims	11, 16-18, 23, 25-30
Inventive step (IS)	Yes:	Claims	3-7, 9, 10
	No:	Claims	1, 2, 8, 11-30
Industrial applicability (IA)	Yes:	Claims	1-30
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following document/s/:

D1: KATER, M.M., ET AL.: 'multiple AGAMOUS homologs from cucumber and petunia differ in their ability to induce reproductive organ fate' THE PLANT CELL, vol. 10, February 1998 (1998-02), pages 171-182, XP002094414

D2: GOODALL, I., ET AL.: 'contribution of high-performance liquid chromatographic analysis of carbohydrates to authenticity testing of honey' JOURNAL OF CHROMATOGRAPHY, vol. 706, 1995, pages 353-359, XP004038886

D3: Database BIOSIS, Abstract, PICARD-NAZOU, A. L. ET AL.: "Foraging behaviour of honey bees (Apis mellifera L.) on transgenic oilseed rape (Brassica napus L. var. oleifera)". Transgenic Research, vol. 4, 1995, pages 270-276.

The document D3 was not cited in the international search report. A copy of the document is appended hereto.

2. The present application does not meet the requirements set forth in Article 33(2) PCT because the subject-matter of claims 11, 16-18, 23 and 25-28 is not new in respect of the prior art as defined in the regulations (Rule 64(1)-(3) PCT).

Document D1 discloses a recombinant double stranded DNA molecule comprising an expression cassette comprising the CaMV 35S promoter, which is functional in the nectaries of plants, fused to the nucleic acid coding for the MADS box transcription factor pMADS3 from *Pethunia x hybrida* (page 175, column 1, line 1). pMADS3 is expressed in the nectaries during late stage pistil development (Figure 3c; page 173, column 2, lines 6-10). The amino acid sequence of pMADS3 is 76% identical to the sequence of the claimed protein FBP15 having SEQ ID: NO 2. D1

further discloses transgenic plants ectopically expressing pMADS3.

The disclosure in D1 anticipates all the features of the subject-matter of claim 11, 16-18, 23 and 25-28 which relate to an expression cassette comprising a promoter functional in nectaries of plants fused to a DNA sequence encoding a protein.

3. The present application does not meet the requirements set forth in Article 33(2) PCT because the subject-matter of claim 21, 29 and 30 is not new in respect of the prior art as defined in the regulations (Rule 64(1)-(3) PCT).

D2 discloses a process for isolating oligosaccharides from the honey of different plant species. This process anticipates all the technical features of the subject-matter of claim 21.

D3 discloses nectar having a modified composition from transgenic oilseed rape (abstract). The nectar being collected by honey bees, it appears that D3 implicitly discloses honey as defined in claims 29 and 30.

4. The subject-matter of claims 1, 2, 8, 12-15, 19, 20, 22 and 24 of the present application does not meet the requirements of Article 33(3) PCT with regard to inventive step.

The applicant formulates the problem as the provision of means and methods to produce metabolites in honey.

As a precondition for acknowledging inventive step, the subject-matter of the claims has to provide a solution to the technical problem over the whole of the scope claimed.

In the present application the problem has only been solved in a non-obvious way by providing the novel protein having the amino acid sequence SEQ ID No: 1, the signal peptide having the amino acid sequence SEQ ID No: 9 and the promoter of

the NEC1 gene.

The cited claims are directed to proteins and promoters for which it is clear that they will not solve the problem.

A mere statement that all of the claimed compounds are "functional" is not enough because such a functional feature is too vague and does not provide sufficient instructions to allow the skilled person to reduce into practice the compounds which have the necessary activity without having to resort to inventive skills.

The above objection can only be met by restricting the scope to those compounds that can be shown to solve the problem posed, i.e. to the compounds having the essential structural feature which is the amino acid or nucleic acid sequence responsible for targeting metabolites to honey.

Re Item VIII

Certain observations on the international application

1. Claims 1, 2, 8, 11-20, 22-28 do not meet the requirements of Article 6 PCT in that the subject-matter for which protection is sought is not supported over its whole scope. The claims define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem.

   Furthermore, the application does not meet the requirements of Article 5 PCT with regard to sufficiency of disclosure (enablement) because only a small number of means of arriving at the solution is disclosed, while all possible means are claimed.

   Note that the concept of support (Article 6 PCT) is not simply a matter of formal concordance between claims and description. It is a substantive requirement reflecting the principle that the scope of an applicant's claim should be justified. Similarly, sufficiency of disclosure (Article 5 PCT) is not met on the basis of a limited and not easily generalisable disclosure.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/NL99/00453

2. Claims 1, 2, 5 and 9 do not meet the requirements of Article 6 PCT because the expression "homology" renders the scope of the claimed subject-matter unclear. It should be replaced in the claims by the definition of the percentage of similarity of the claimed sequence (see for example, in the description on page 12, line 12-14).

3. The isolated DNA sequences of claims 3 and 9 are defined by erroneous SEQ ID numbers, thereby rendering the claims unclear (Article 6 PCT). ?

CLAIMS

1. An isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:1, or a protein that has at least 60% homology to the amino acid sequence given in SEQ ID NO:1.

2. An isolated DNA sequence according to claim 1, wherein the nectary-specific expressed sequence has:
   a) a nucleotide sequence given in SEQ ID NO:4, or
   b) a nucleotide sequence which hybridises with (a) or with a fragment of (a) under the following conditions: pre-hybridisation for 1h at about 65 °C in a solution of Church and Gilbert, comprising 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS, followed by hybridisation in the same solution for 18h at about 65 °C, followed by washing three times in 0.1 x SSC, 0.1% SDS at about 65 °C for 30 min., or
   c) a nucleotide sequence that has at least 85% homology to the nucleotide sequence of a).

3. An isolated DNA sequence according to claim 1 or 2, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:7, or a functional fragment thereof having promoter activity.

4. An isolated DNA sequence encoding a protein comprising the amino acid sequence given in SEQ ID NO:1, or a protein having at least 60% homology with the amino acid sequence given in SEQ ID NO:1, which protein, when ectopically expressed, plays a role in sugar metabolism, the expression of the DNA sequence being predominantly confined to the nectaries of a plant.

5. An isolated DNA sequence according to claim 4 having:
   a) a nucleotide sequence given in SEQ ID NO:4, or

b) a nucleotide sequence that hybridises with the nucleotide sequence of (a) or with a fragment of (a) under the hybridisation conditions as defined in claim 2, or

c) a nucleotide sequence that has at least 85% homology to the nucleotide sequence of a).

6. An isolated DNA sequence that results from the sequence shown in SEQ ID NO:4 by insertion, deletion or substitution of one or more nucleotides, including naturally occurring variations or variations introduced by targeted mutagenesis or recombination, wherein the DNA sequence encodes a protein exhibiting the same function as the protein according to claim 4.

7. An isolated DNA sequence according to claim 4 having a nucleotide sequence given in SEQ ID NO:4, said sequence being produced by current DNA synthesis techniques.

8. An isolated DNA sequence comprising the coding region for a signal peptide, wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein that is expressed in nectarics, targeting of the protein to nectar.

9. An isolated DNA sequence according to claim 8, having:

a) a nucleotide sequence given in SEQ ID NO:6 obtained from a plant of *Calluna vulgaris*, or

b) a nucleotide sequence that hybridises with the nucleotide sequence given in a), under the hybridisation conditions as defined in claim 2, or

c) a nucleotide sequence that has at least 95% homology to the nucleotide sequence of a).

10. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in plants,

ii) a DNA sequence coding for a protein as defined in any of

claims 4 to 7 which is fused to the promoter sequence in sense or antisense orientation, and optionally

iii) a signal sequence functional in plants for the transcription determination and polyadenylation of an RNA molecule.

11. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in nectaries of plants,

ii) a DNA sequence coding for a protein which is fused to the promoter sequence in sense or antisense orientation, and optionally

iii) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

12. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in nectaries of plants,

ii) a DNA sequence encoding a protein which is fused to the promoter,

iii) a DNA sequence encoding a signal peptide that targets the recombinant protein to nectar, which is translationally fused to the DNA sequence encoding the recombinant protein, and optionally

iv) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

13. A recombinant double-stranded DNA molecule according to claim 11 or 12 wherein the promoter is as defined in any of claims 1-3.

14. A recombinant double-stranded DNA molecule according to claim 12 or 13 wherein the DNA sequence encoding a signal peptide is as defined in claim 8 or 9.

15. A process for producing a transgenic plant exhibiting excretion of a recombinant protein in its nectar, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 12 to 14, wherein the recombinant protein is excreted in nectar,

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

16. A process for producing a transgenic plant exhibiting a modified nectar composition, comprising:
   i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 11 to 14, wherein the recombinant protein interferes with metabolic pathways in the nectaries,
   ii) regenerating plants from the transgenic cell, and
   iii) selecting transgenic plants.

17. A process for producing a transgenic plant exhibiting a modified nectar secretion, comprising:
   i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 11 to 14, wherein the recombinant protein interferes with sink strength of nectaries,
   ii) regenerating plants from the transgenic cell, and
   iii) selecting transgenic plants.

18. A process for producing a transgenic plant exhibiting a modified nectary development, comprising:
   i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in claims 11 or 14, wherein the recombinant protein interferes with the development of nectaries,
   ii) regenerating plants from the transgenic cell, and
   iii) selecting transgenic plants.

19. A process for producing honey from modified nectar of transgenic plants, comprising:
   i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule as defined in any of claims 11 to 14, regenerating plants from the transgenic cell, and selecting modified plants exhibiting the excretion of nectar with a modified composition,
   ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey.

20. A process for producing a recombinant gene product from honey, comprising:
   i) producing a transgenic plant by introducing in a plant cell

a recombinant double-stranded DNA molecule as defined in any of claims 12 to 14, regenerating plants from the transgenic cell, and selecting modified plants exhibiting excretion of the recombinant gene product in nectar,

ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey, and

iii) isolating and purifying the gene product from the honey.

21. A process for producing a metabolite from honey, comprising:

i) producing a plant that excretes this metabolite in nectar and which plant has been produced by current breeding and selection methods,

ii) allowing insects, preferably bees, to collect nectar from the selected plants and to process the nectar into honey, and

iii) isolating and purifying the metabolite from the honey.

22. Micro organisms containing DNA sequences according to one or more of claims 1 to 9.

23. Micro organisms containing recombinant DNA molecules according to any of claims 10 to 14.

24. A plant cell or plant cell culture transformed with one or more DNA sequences according to claims 1 to 9.

25. A plant cell or plant cell culture transformed with recombinant DNA molecules according to any of 10 to 14.

26. A plant consisting escentially of the plant cells of claims 24 or 25.

27. A transgenic plant obtained by the process of any of claims 15 to 18.

28. Seeds, tissue culture, plant parts or progeny plants derived from a transgenic plant according to claim 27.

29. Honey obtained from nectar from transgenic plants, which nectar has a modified composition.

30. Honey obtained from nectar from transgenic plants, which nectar comprises a recombinant gene product.

Claims

1. An isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO: 1, or a homologous protein.

2. An isolated DNA sequence according to claim 1, wherein the nectary-specific expressed sequence has:
a) a nucleotide sequence given in SEQ ID NO:4, or
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

3. An isolated DNA sequence according to claim 1 or 2, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:7, or a functional fragment thereof having promoter activity.

4. An isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:2, or a homologous protein.

5. An isolated DNA sequence according to claim 4, wherein the nectary-specific expressed sequence has:
a) a nucleotide sequence given in SEQ ID NO:5, or
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

6. An isolated DNA sequence according to claim 4 or 5, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:8 or a functional fragment thereof having promoter activity.

7. An isolated DNA sequence encoding a protein comprising the amino acid sequence given in SEQ ID NO:1, or a homologous protein, the expression of the DNA sequence being predominantly confined to the nectaries of a plant.

8. An isolated DNA sequence according to claim 7 having:
a) a nucleotide sequence given in SEQ ID NO:4, or
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

9. An isolated DNA sequence that results from the sequence shown in SEQ ID NO:4 by insertion, deletion or substitution of one or more nucleotides, including naturally occurring variations or variations introduced by targeted mutagenesis or recombination.

10. An isolated DNA sequence according to claim 7 having a nucleotide sequence given in SEQ ID NO:4, said sequence being produced by current DNA synthesis techniques.

11. An isolated DNA sequence encoding a protein comprising the amino acid sequence given in SEQ ID NO:2, or a homologous protein, the expression of the DNA sequence being predominantly confined to the nectaries of a plant.

12. An isolated DNA sequence according to claim 11, having:
a) a nucleotide sequence given in SEQ ID NO:5, or
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

13. An isolated DNA sequence that results from the sequence shown in SEQ ID NO:5 by insertion, deletion or substitution of one or more nucleotides, including naturally occurring variations or variations introduced by targeted mutagenesis or recombination.

14. An isolated DNA sequence according to claim 11 having a nucleotide sequence given in SEQ ID NO:5, said sequence being produced by current DNA synthesis techniques.

15. Any sequence that encodes a nectary-specific MADS box gene.

16. An isolated DNA sequence comprising the coding region for a signal peptide, wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein that is expressed in nectaries, targeting of the protein to nectar.

17. An isolated DNA sequence according to claim 16, comprising the nucleotide sequence given in SEQ ID NO: 6 obtained from a plant of *Calluna vulgaris*, or a nucleotide sequence obtainable by hybridisation with the nucleotide sequence given in SEQ ID NO:6.

18. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in plants,

ii) a DNA sequence coding for a protein as defined in any of claims 7 to 15 which is fused to the promoter sequence in sense or antisense orientation, and optionally

iii) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

19. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in nectaries of plants,

ii) a DNA sequence coding for a protein which is fused to the promoter sequence in sense or antisense orientation, and optionally

iii) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

20. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

i) a promoter functional in nectaries of plants,

ii) a DNA sequence encoding a protein which is fused to the promoter,

iii) a DNA sequence encoding a signal peptide that targets the recombinant protein to nectar, which is translationally fused to the DNA sequence encoding the recombinant protein, and optionally

iv) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

21. A recombinant double-stranded DNA molecule according to claim 19 or 20 wherein the promoter is as defined in any of claims 1-6.

22. A recombinant double-stranded DNA molecule according to claim 20 or 21 wherein the DNA sequence encoding a signal peptide is as defined in claim 16 or 17.

23. A process for producing a transgenic plant exhibiting excretion of a recombinant protein in its nectar, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 20 to 22, wherein the recombinant protein is excreted in nectar

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

24. A process for producing a transgenic plant exhibiting a modified nectar composition, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 19 to 22,

wherein the recombinant protein interferes with metabolic pathways in the nectaries,

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

25. A process for producing a transgenic plant exhibiting a modified nectar secretion, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 19 to 22, wherein the recombinant protein interferes with sink strength of nectaries

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

26. A process for producing a transgenic plant exhibiting a modified nectary development, comprising:

i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in claims 19 or 22, wherein the recombinant protein interferes with the development of nectaries

ii) regenerating plants from the transgenic cell, and

iii) selecting transgenic plants.

27. A process for producing honey from modified nectar of transgenic plants, comprising:

i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule as defined in any of claims 19 to 22, regenerating plants from the transgenic cell, and selecting modified plants exhibiting the excretion of nectar with a modified composition,

ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey.

28. A process for producing a recombinant gene product from honey, comprising:

i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule as defined in any of claims 20 to 22, regenerating plants from the transgenic cell, and selecting modified plants exhibiting excretion of the recombinant gene product in nectar,

ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey, and

iii) isolating and purifying the gene product from the honey.

29. A process for producing a metabolite from honey, comprising:

i) producing a plant that excretes this metabolite in nectar and which plant has been produced by current breeding and selection methods,

ii) allowing insects, preferably bees, to collect nectar from the selected plants and to process the nectar into honey, and

iii) isolating and purifying the metabolite from the honey.

30. Micro organisms containing DNA sequences according to one or more of claims 1 to 17.

31. Micro organisms containing recombinant DNA molecules according to any of claims 18 to 22.

32. A plant cell or plant cell culture transformed with one or more DNA sequences according to claims 1 to 17.

33. A plant cell or plant cell culture transformed with recombinant DNA molecules according to any of 18 to 22.

34. A plant consisting essentially of the plant cells of claims 32 or 33.

35. A transgenic plant obtained by the process of any of claims 23 to 26.

36. Seeds, tissue culture, plant parts or progeny plants derived from a transgenic plant according to claim 35.

37. Honey obtained from nectar from transgenic plants, which nectar has a modified composition.

38. Honey obtained from nectar from transgenic plants, which nectar comprises a recombinant gene product.